
به نام خداوند مهر گستر مهربان

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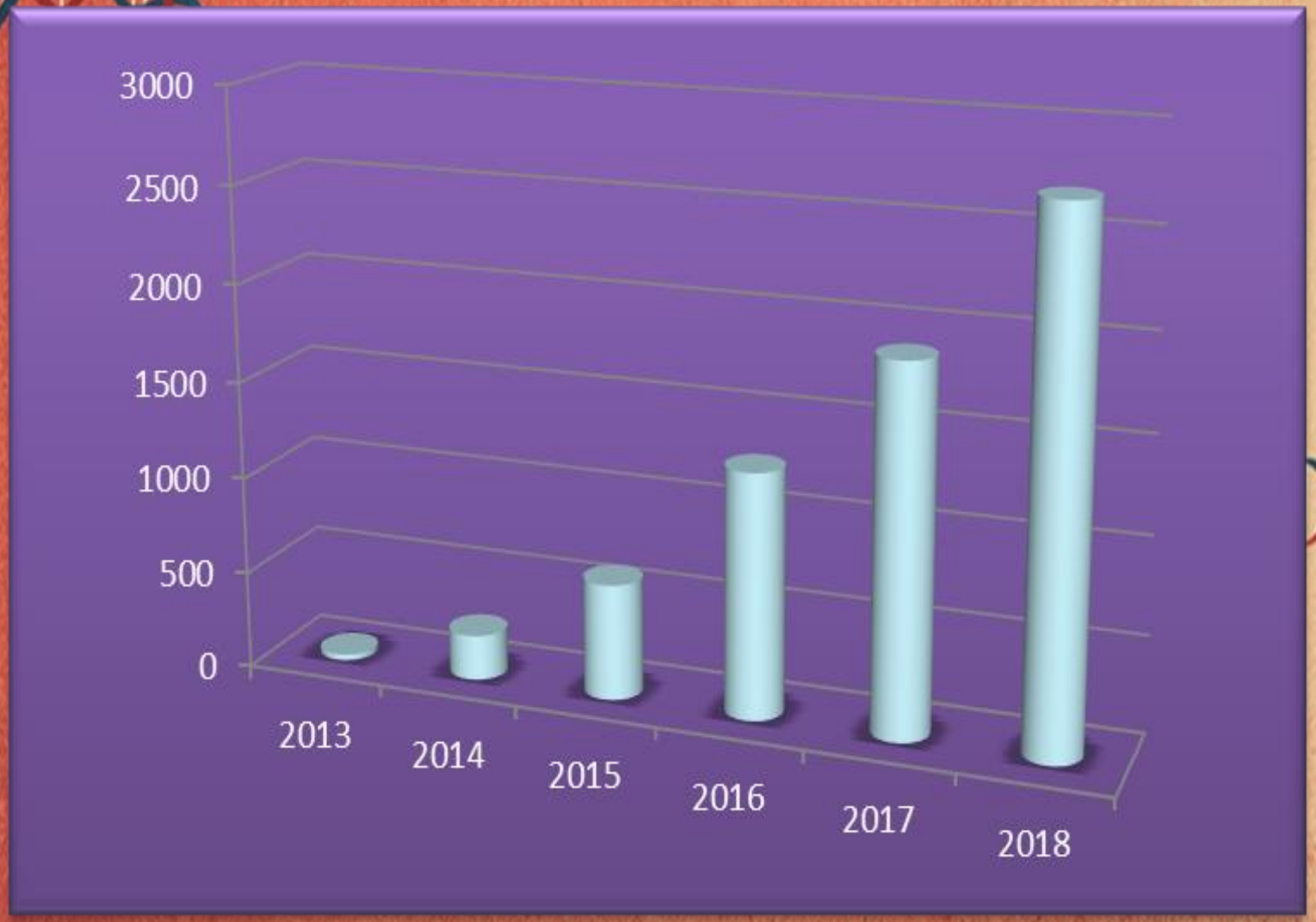
Research of CRISPR Trend

(Pubmed-5th January2018)



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- Off target detection approaches
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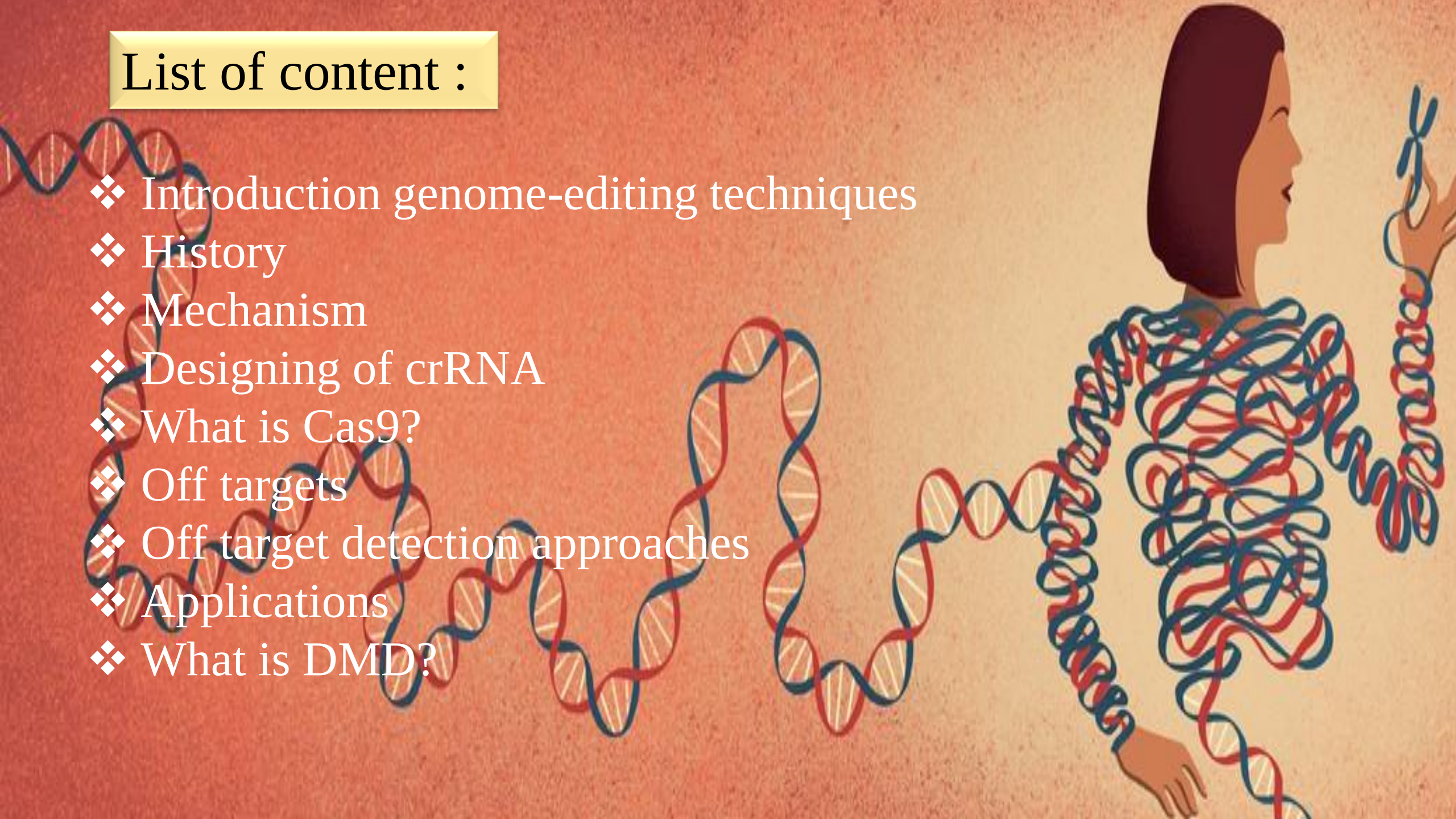
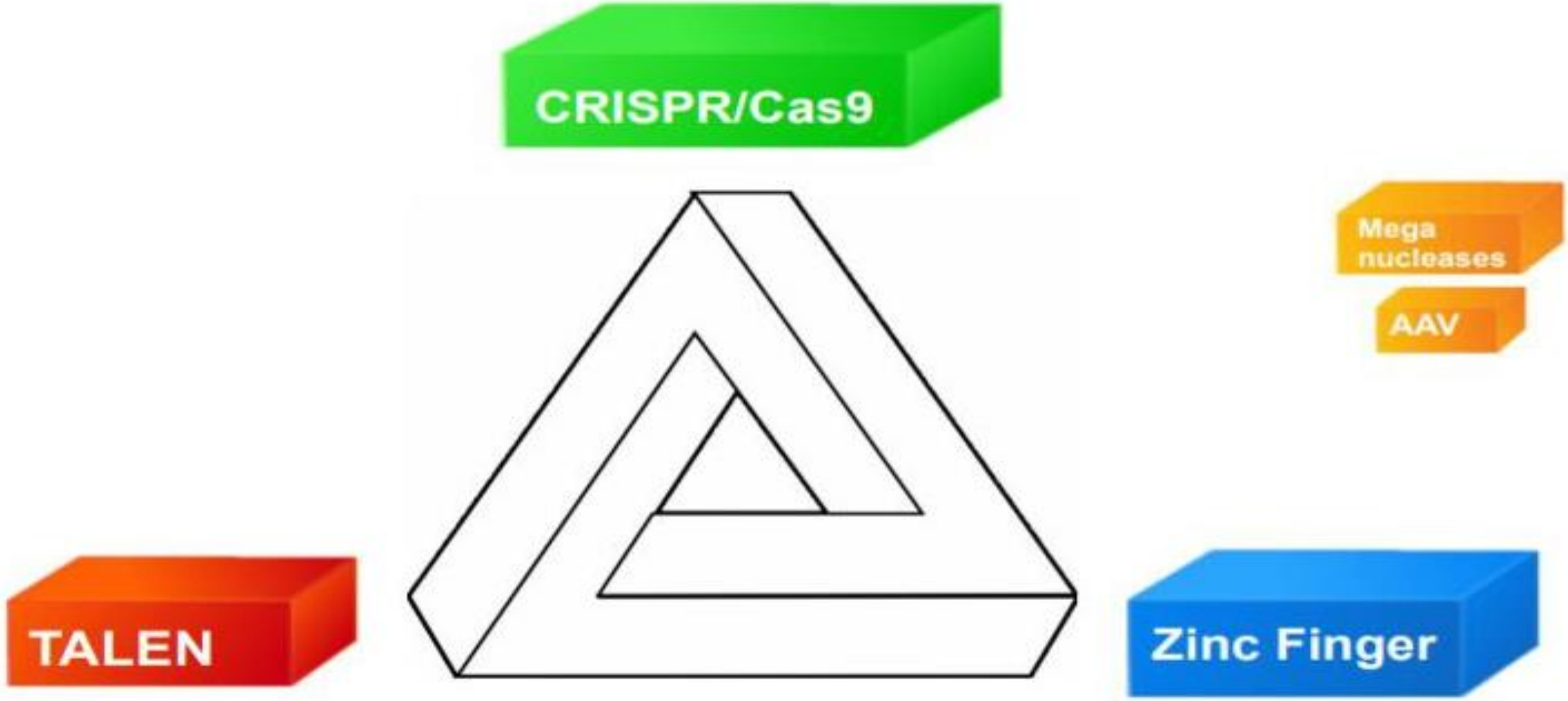
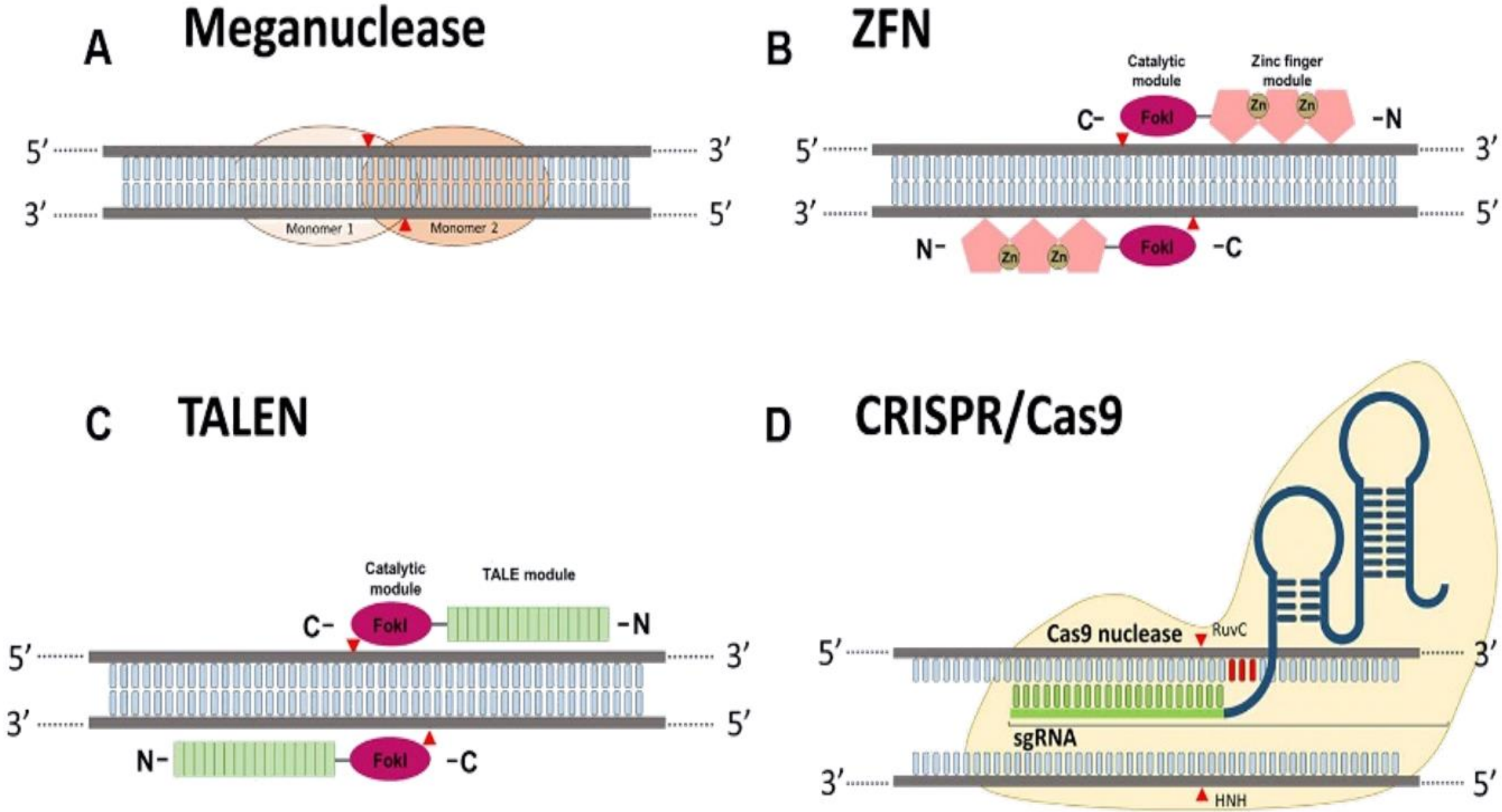
- What is DMD?



The triumvirate of genome editing



Read:Gaj,Gersbatch,barbas3:Trends in Biotechnplogy,July2013,vol31,No.7


A
Meganuclease
5′
3′
3′
5′
Monomer 1
Monomer 2
B
ZFN
Catalytic module
Zinc finger module
C-
FokI
Zn
Zn
-N
5′
3′
3′
5′
N-
Zn
Zn
FokI
-C
C
TALEN
Catalytic module
TALE module
C-
FokI
-N
5′
3′
3′
5′
N-
FokI
-C
D
CRISPR/Cas9
Cas9 nuclease
RuvC
5′
3′
sgRNA
3′
5′
HNH

CRISPR

C: Clustered

R: regularly

I: interspaced

S: short

P: palindromic

R: repeat

Why is CRISPR/Cas9 better ?


Jennifer Doudna
Biochemical characterization of Cas9-mediated cleavage
Dr. Doudna was trying to figure out exactly how this happened.

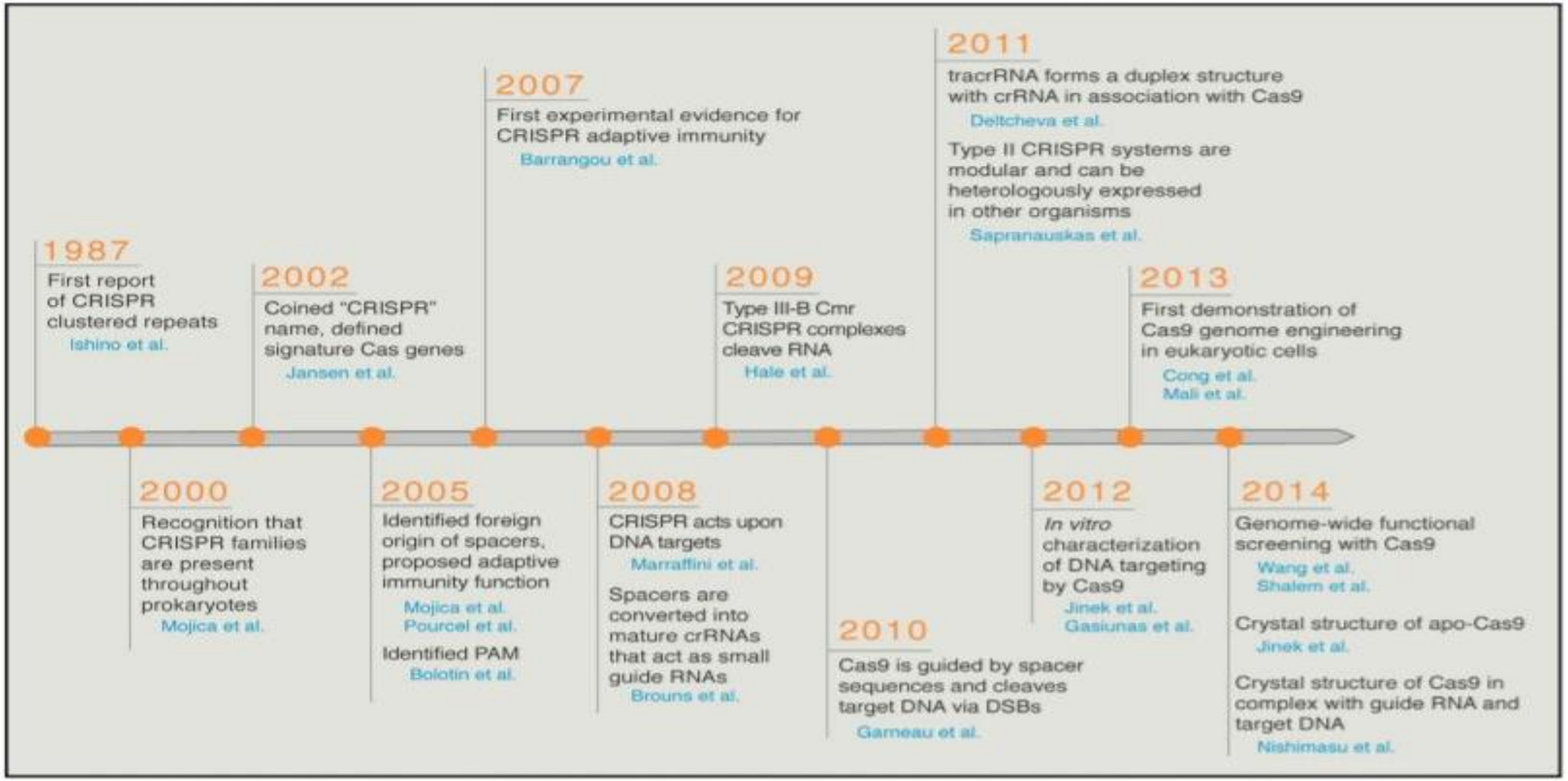
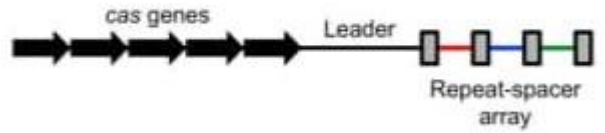


Figure: Hsu, Lander, Zhang: Development and applications of CRISPR-Cas9 for Genome Engineering:Cell157, June5, 2014

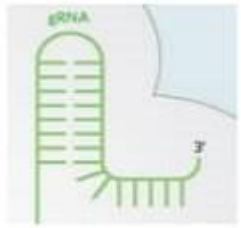
CRISPR loci and Cas nucleases nomenclature

CRISPR: Clustered Regulatory Interspaced Palindromic repeats
Loci in %40 of bacteria and %90 of archea

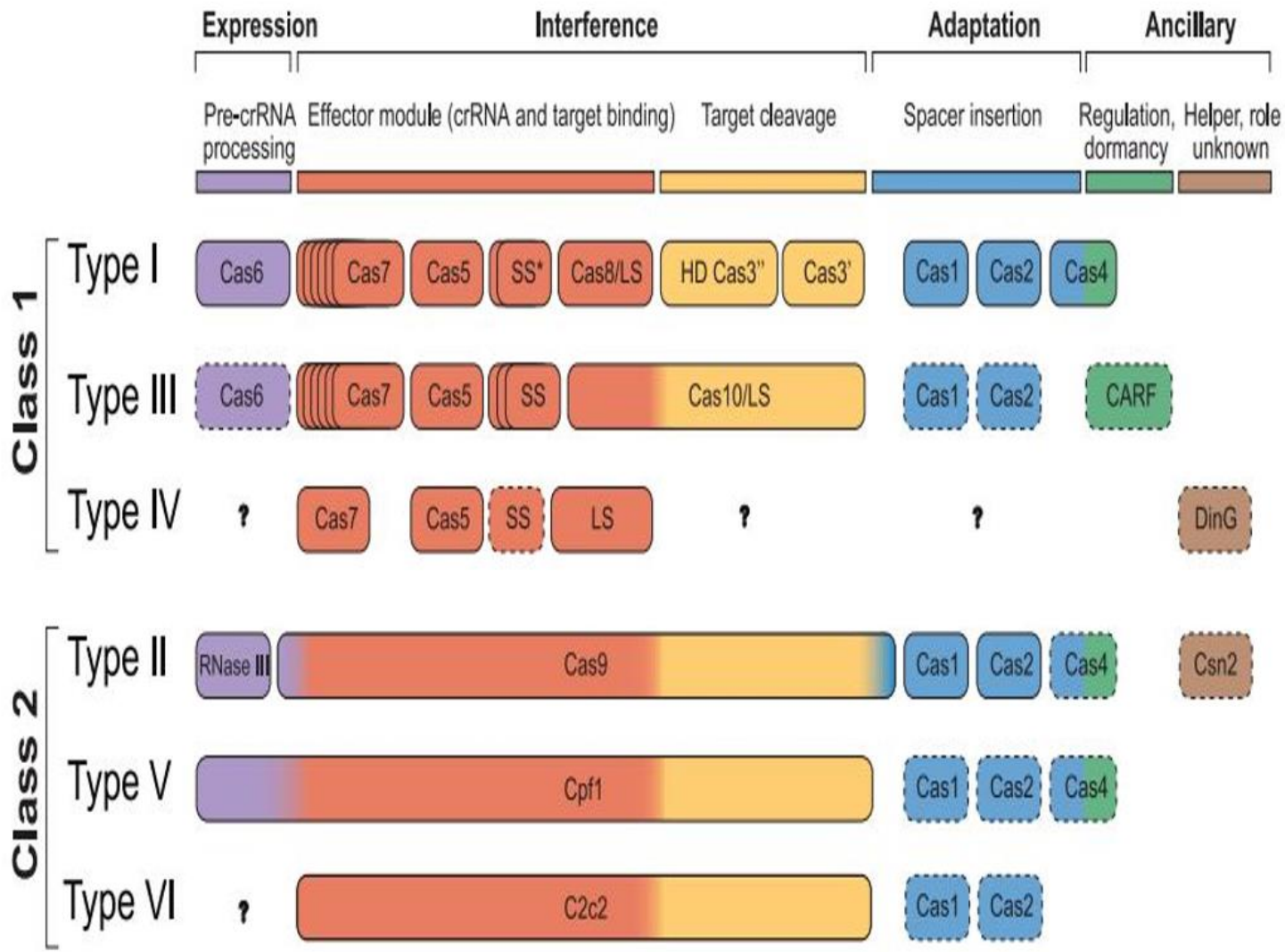


Cas9: CRISPR associated protein9
A nuclease, an enzyme specialized for cutting DNA

gRNA: a construct/chimera of CRISPR RNA(crRNA) and trans-activating CRISPR RNA(tracrRNA)



PAM: proto spacer adjacent with sequence NGG(any, guanine, guanine) specific to Streptococcus pyogenes


Expression
Interference
Adaptation
Ancillary
Pre-crRNA processing
Effector module (crRNA and target binding)
Target cleavage
Spacer insertion
Regulation, dormancy
Helper, role unknown
Class 1
Type I
Cas6
Cas7
Cas5
SS*
Cas8/LS
HD Cas3''
Cas3'
Cas1
Cas2
Cas4
Type III
Cas6
Cas7
Cas5
SS
Cas10/LS
Cas1
Cas2
CARF
Type IV
?
Cas7
Cas5
SS
LS
?
?
DinG
Class 2
Type II
RNase III
Cas9
Cas1
Cas2
Cas4
Csn2
Type V
Cpf1
Cas1
Cas2
Cas4
Type VI
?
C2c2
Cas1
Cas2

How CRISPR system work as an bacterial immune system

Action Of CRISPR in bacteria

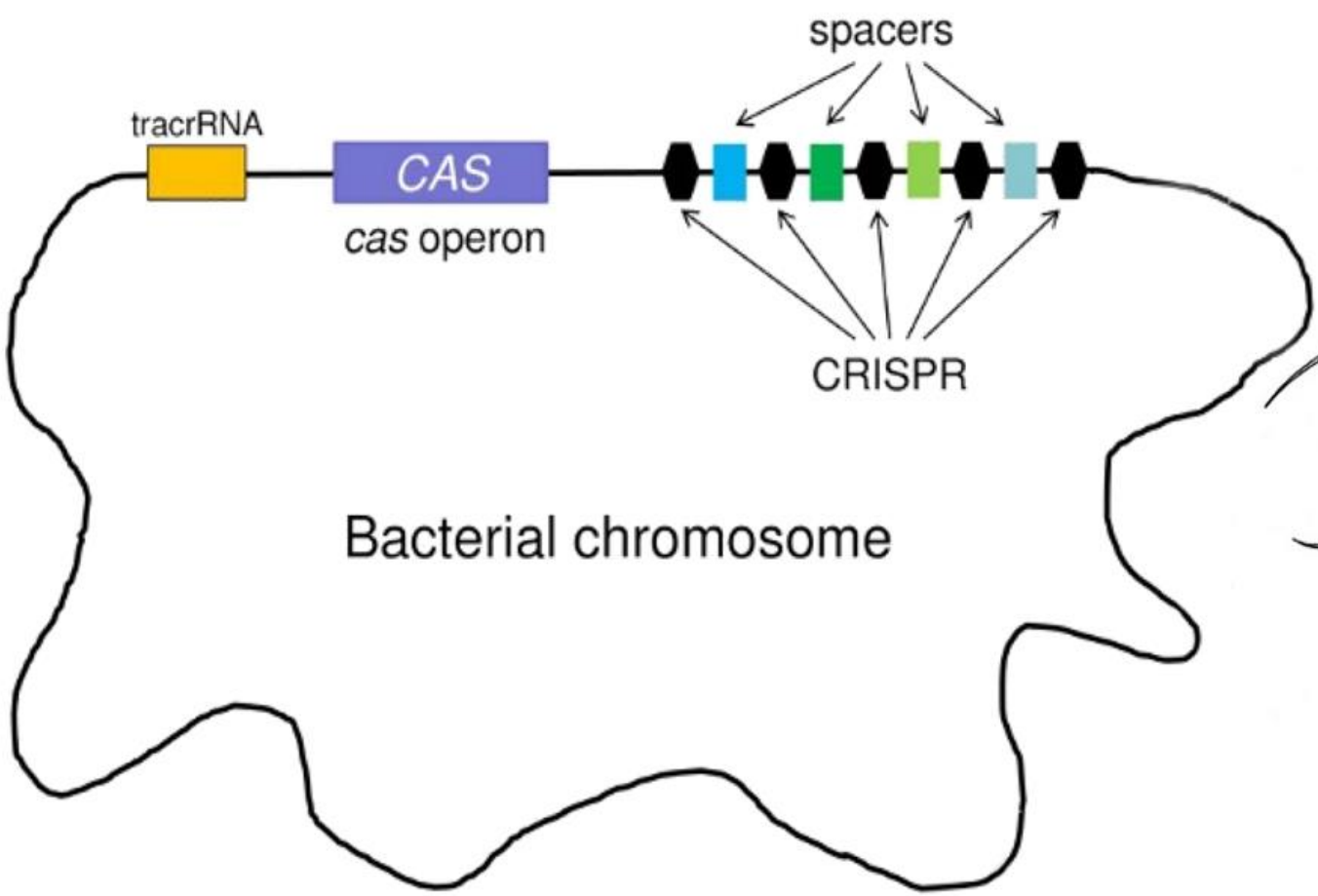
The CRISPR immune system works to protect bacteria from repeated viral attack via three basic steps:

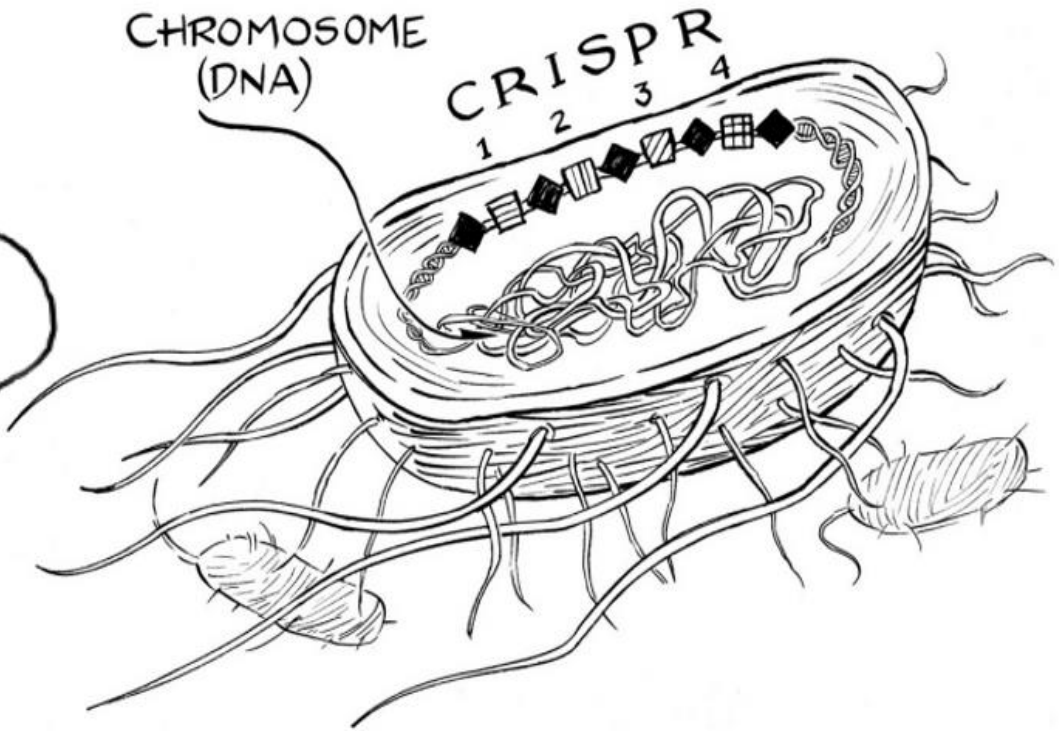
(1) Adaptation

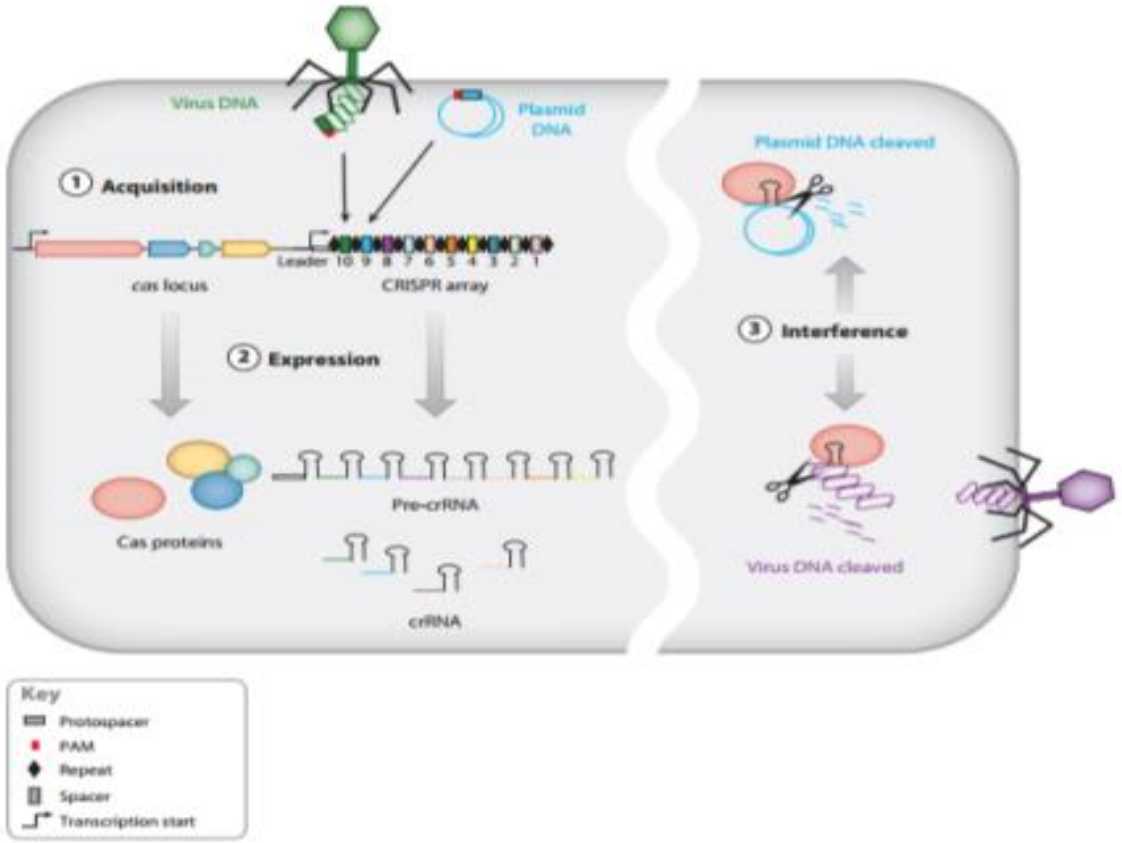
(2) Production of crRNA

(3) Targeting

The CRISPR locus in bacteria



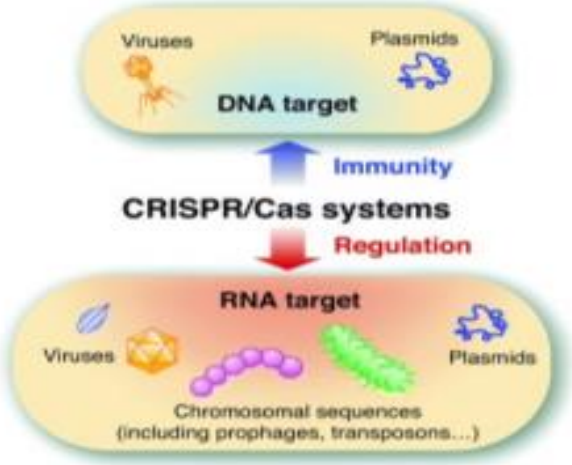




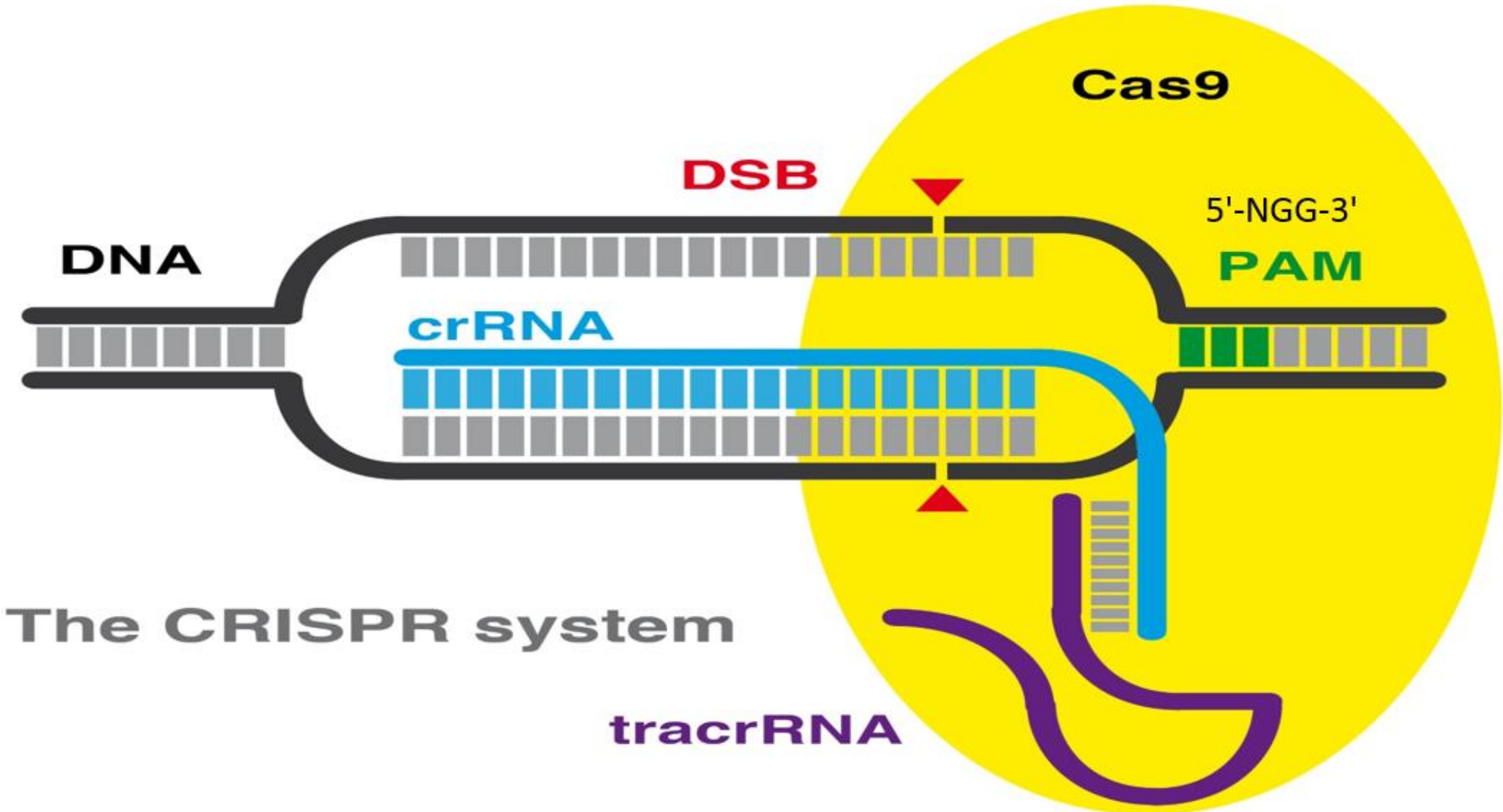
(1) acquisition of foreign DNA

(2) synthesis and maturation of CRISPR RNA (crRNA) followed by formation of RNA-Cas nuclease protein complexes

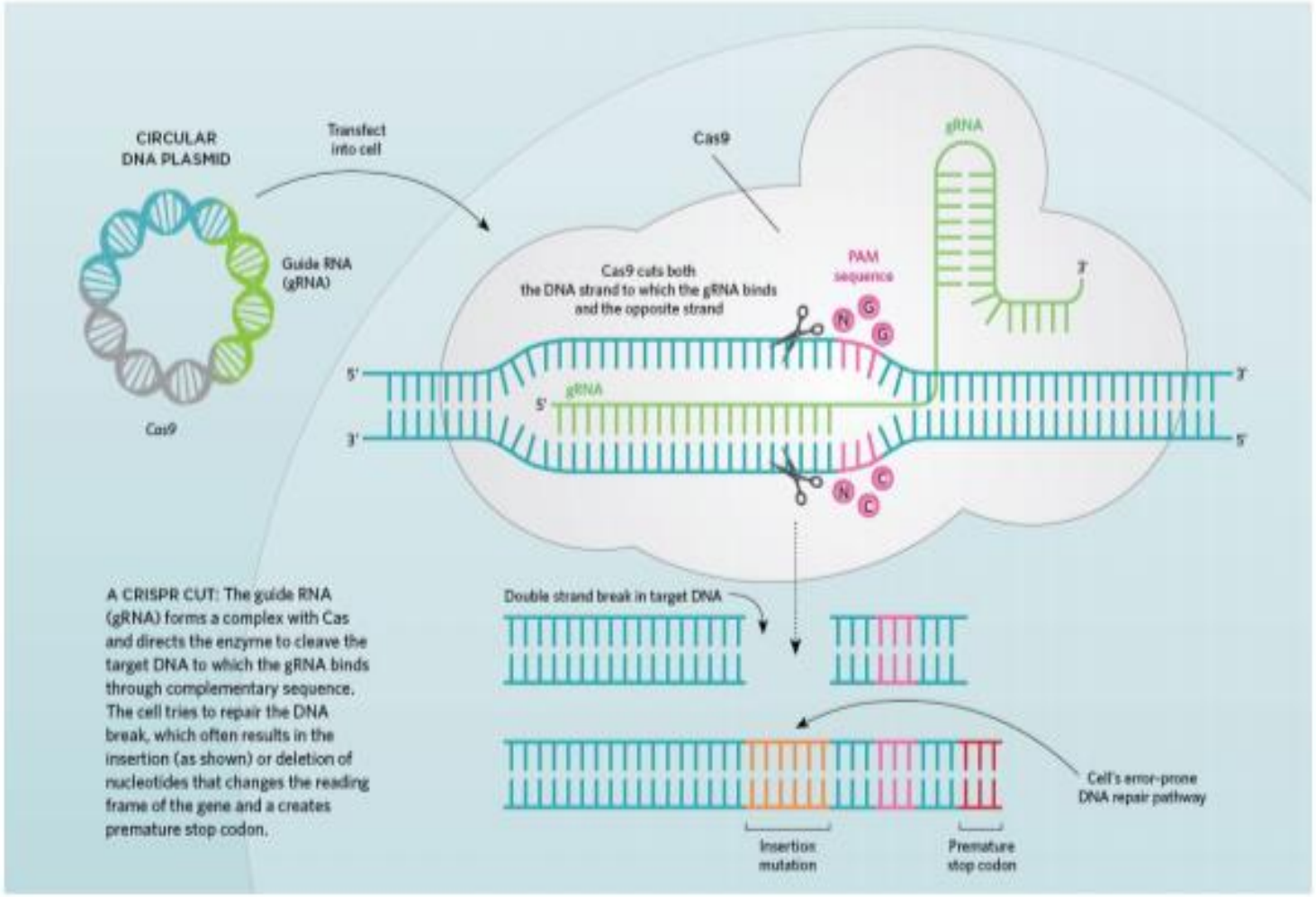
(3) target recognition by crRNA and destruction of foreign DNA by Cas nuclease cleavage



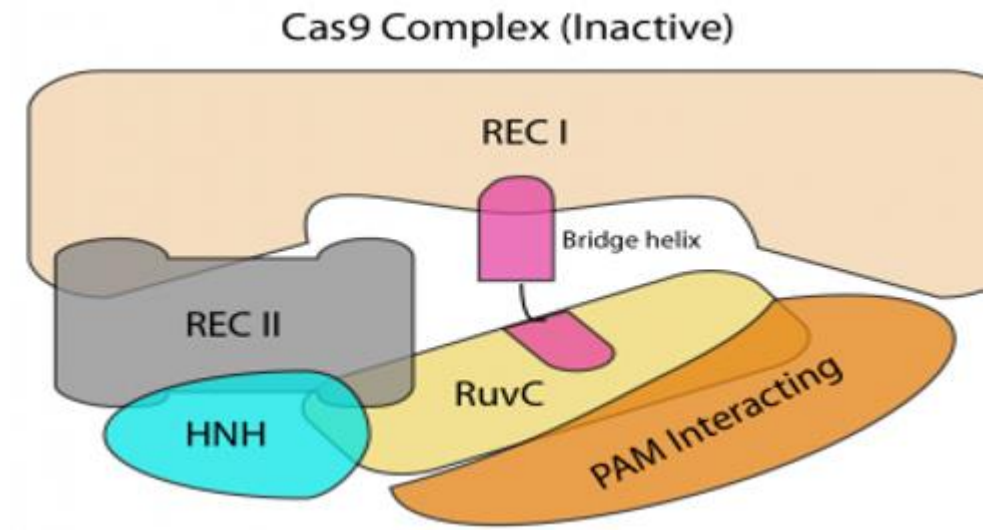
Figures: Annu.Rev.Genet.2011.45:273-97 and Horvath: science (2010) vol.327;167-170: CRISPR/Cas , the immune system of Bacteria and Archaea

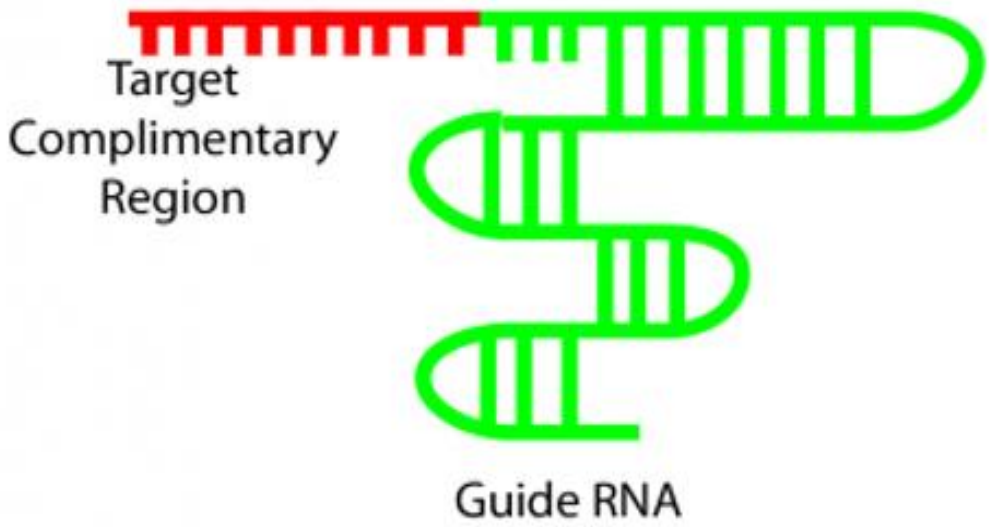

Cas9
DSB
5'-NGG-3'
PAM
DNA
crRNA
The CRISPR system
tracrRNA

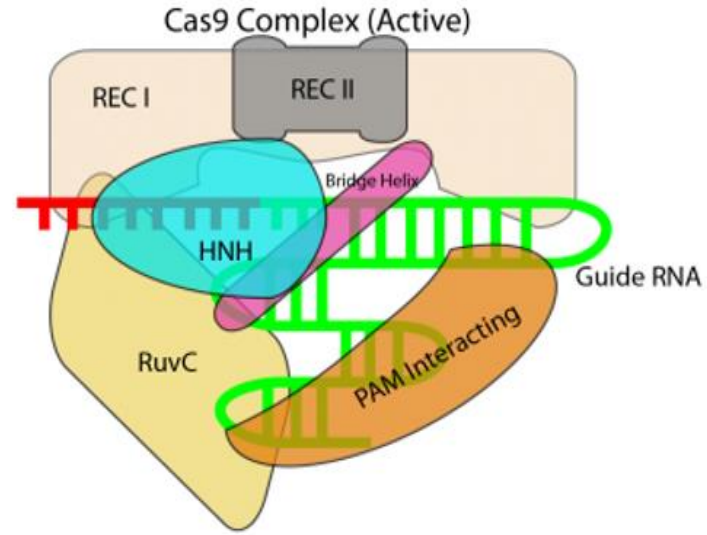
CRISPR/Cas9 process includes a targeted double strand break

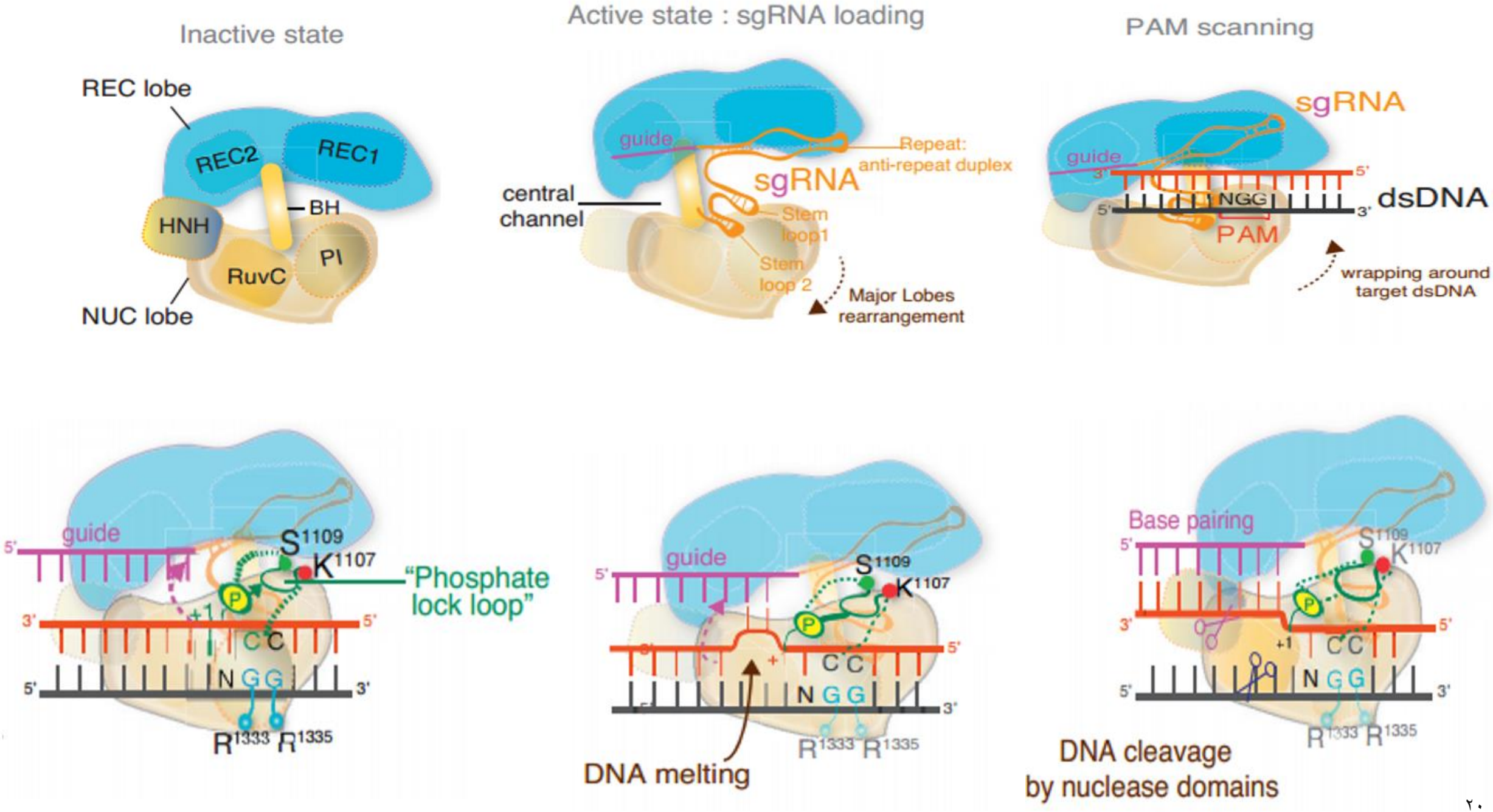


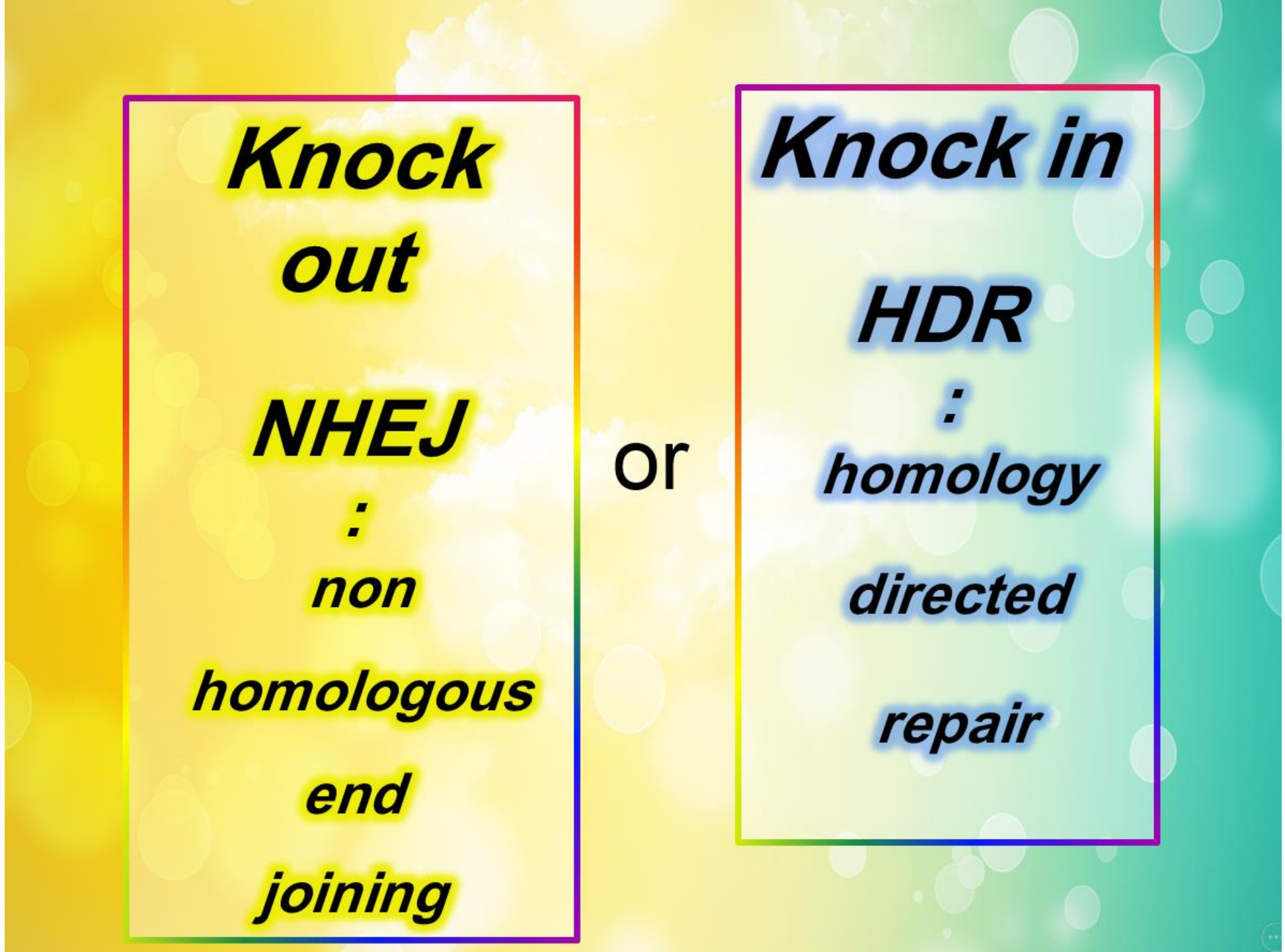
The gRNA forms a complex with Cas directs the enzyme to cleave the target DNA to switch the gRNA binds through complementarysequences


Cas9 Complex (Inactive)
REC I
Bridge helix
REC II
RuvC
HNH
PAM Interacting


Target
Complimentary
Region
Guide RNA


Cas9 Complex (Active)
REC I
REC II
Bridge Helix
HNH
Guide RNA
RuvC
PAM Interacting


Inactive state
REC lobe
REC2
REC1
BH
HNH
RuvC
PI
NUC lobe
Active state : sgRNA loading
guide
Repeat:
anti-repeat duplex
sgRNA
central
channel
Stem
loop1
Stem
loop 2
Major Lobes
rearrangement
PAM scanning
sgRNA
guide
3'
5'
NGG
5'
3'
dsDNA
PAM
wrapping around
target dsDNA
guide
5'
S1109
K1107
"Phosphate
lock loop"
P
+1
3'
5'
C C
N G G
5'
3'
R1333 R1335
guide
5'
S1109
K1107
P
3'
5'
C C
N G G
3'
R1333 R1335
DNA melting
Base pairing
5'
S1109
K1107
P
3'
+1
5'
C C
5'
N G G
3'
R1333 R1335
DNA cleavage
by nuclease domains


Knock out
NHEJ
:
non homologous end joining
or
Knock in
HDR
:
homology directed repair

Endogenous DNA repair mechanisms

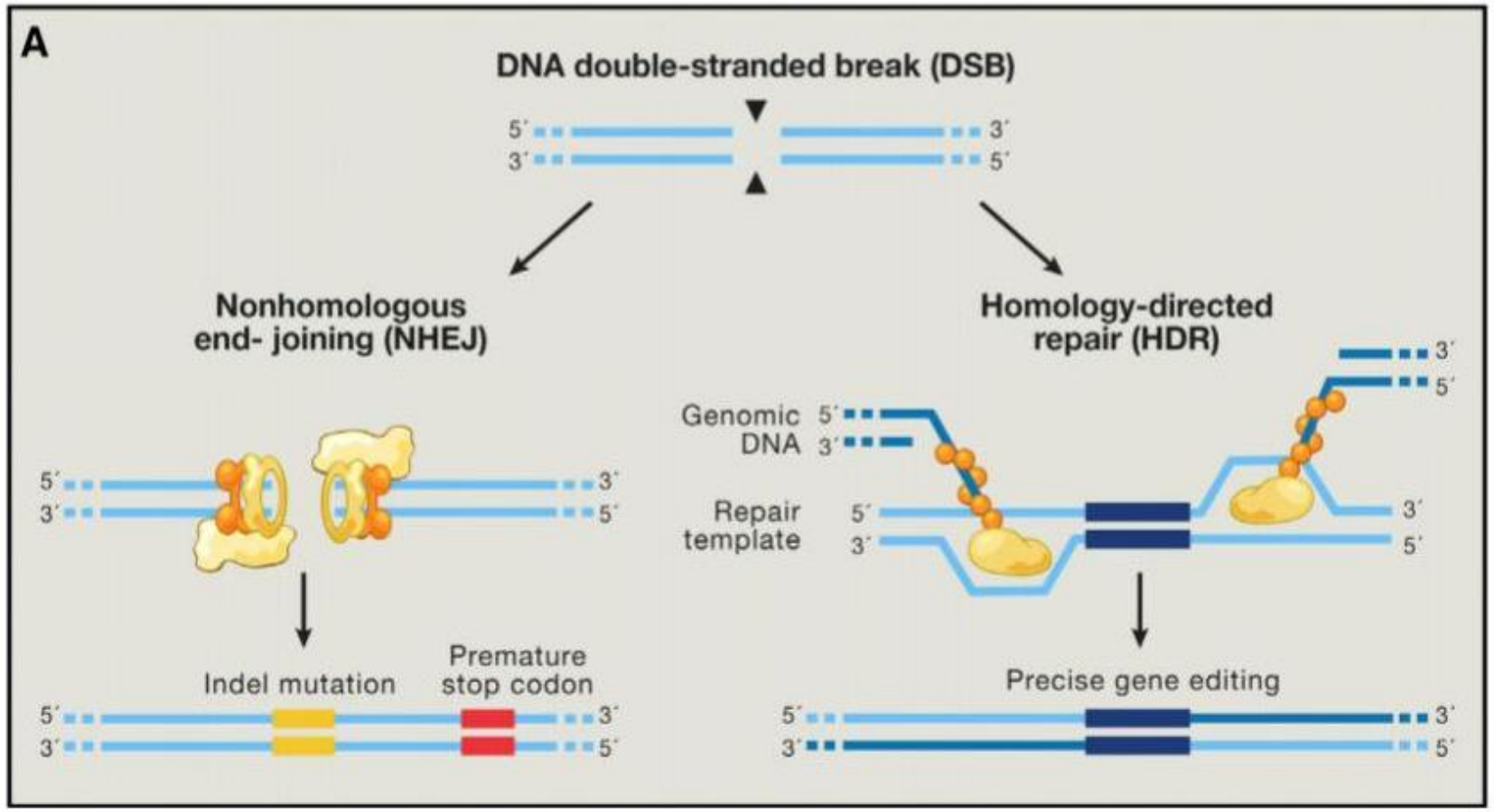
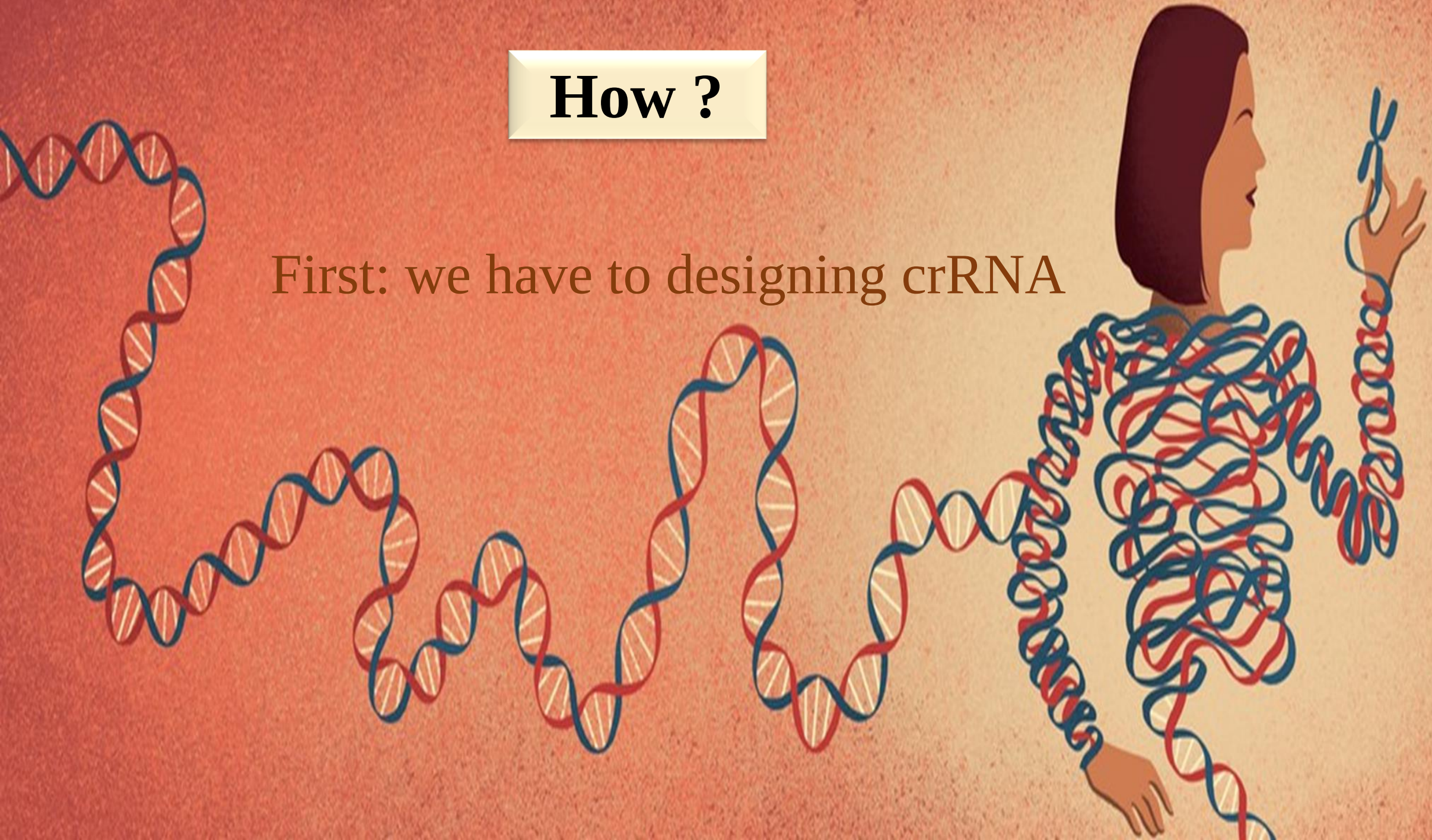
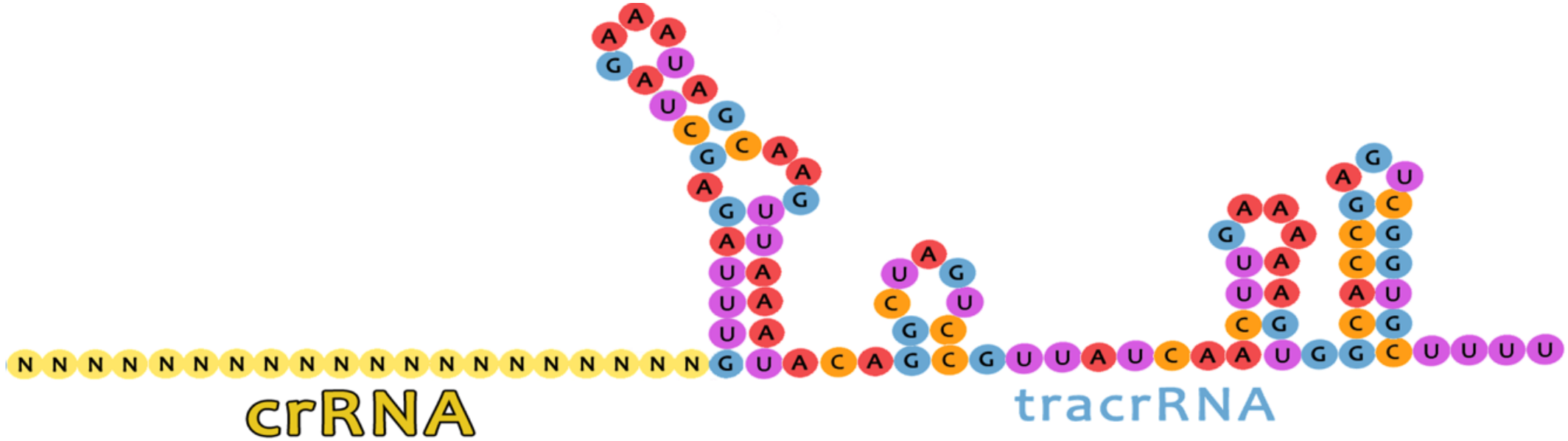


Figure : Hsu ,Lander ,Zhang : development and applications of CRISPR-Cas9 for Genome Engineering: cell157,June 5,2014

How ?

First: we have to designing crRNA




crRNA
tracrRNA


single guide RNA
(sgRNA)
5′–NNNNNNNNNNNNNNNNNNNN
AAGGCUAGUCCGUUAUCAA
3′–UUUU

sgRNA designing tools

- **Optimized CRISPR Design** (Feng Zhang's Lab at MIT/BROAD, USA)
- **sgRNA Scorer** (George Church's Lab at Harvard, USA)
- **sgRNA Designer** (BROAD Institute)
- **ChopChop web tool** (George Church's Lab at Harvard, USA)
- **E-CRISP** (Michael Boutros' lab at DKFZ, Germany)
- **CRISPR Finder** (Wellcome Trust Sanger Institute, Hinxton, UK)
- **RepeatMasker** (Institute for Systems Biology) to double check and avoid selecting target sites with repeated sequences

companies

CARIBOU BIOSCIENCES

editas MEDICINE

Intellia THERAPEUTICS

CRISPR THERAPEUTICS

ToolGen

ERS GENOMICS

TaKaRa Clontech

Broad LIC

life technologies

horizon

Harvard, Broad, ERS LIC

GE Healthcare Dharmacon RNAi

Broad LIC

SIGMA-ALDRICH

Broad LIC

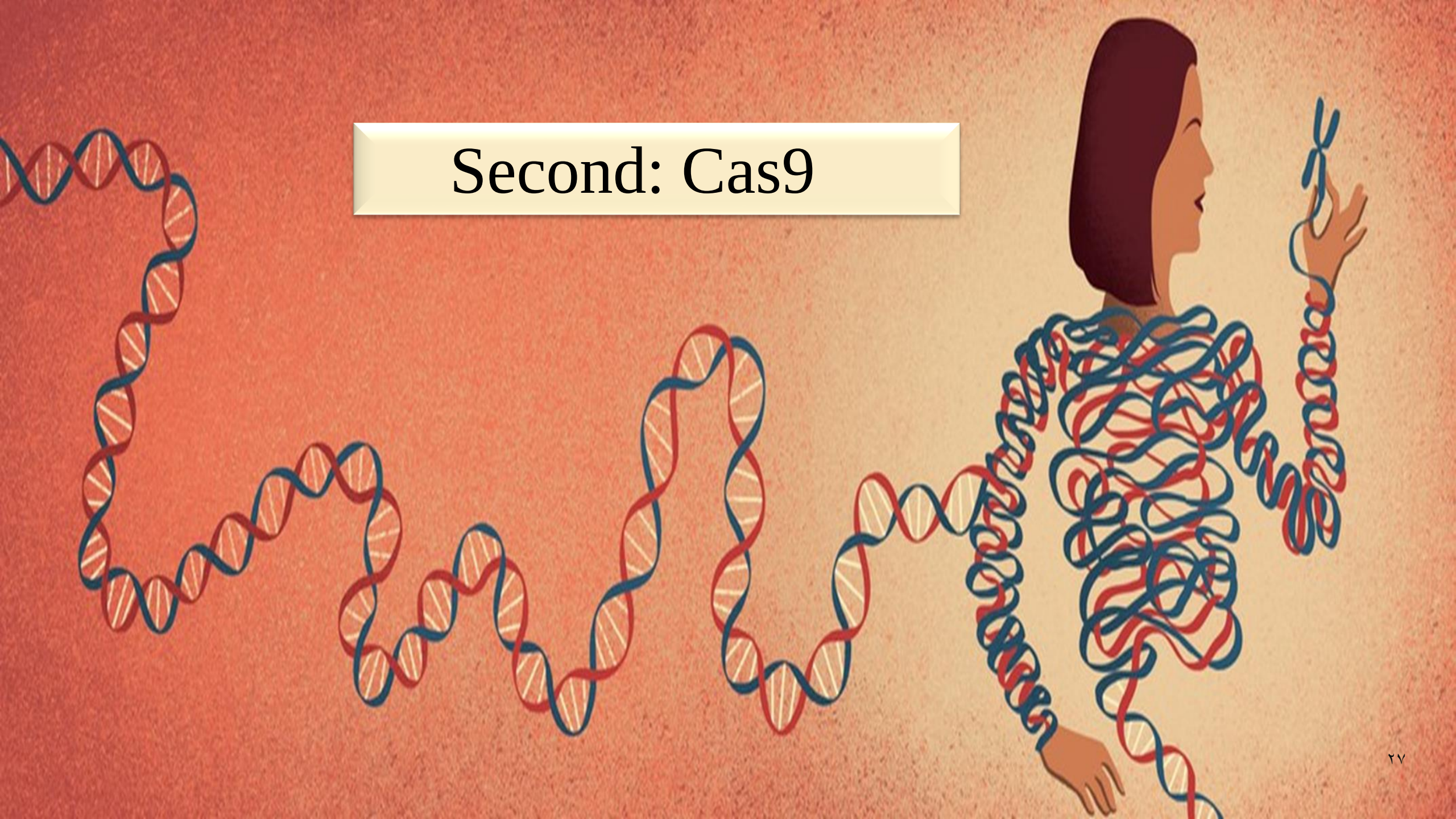
SAGE LABS

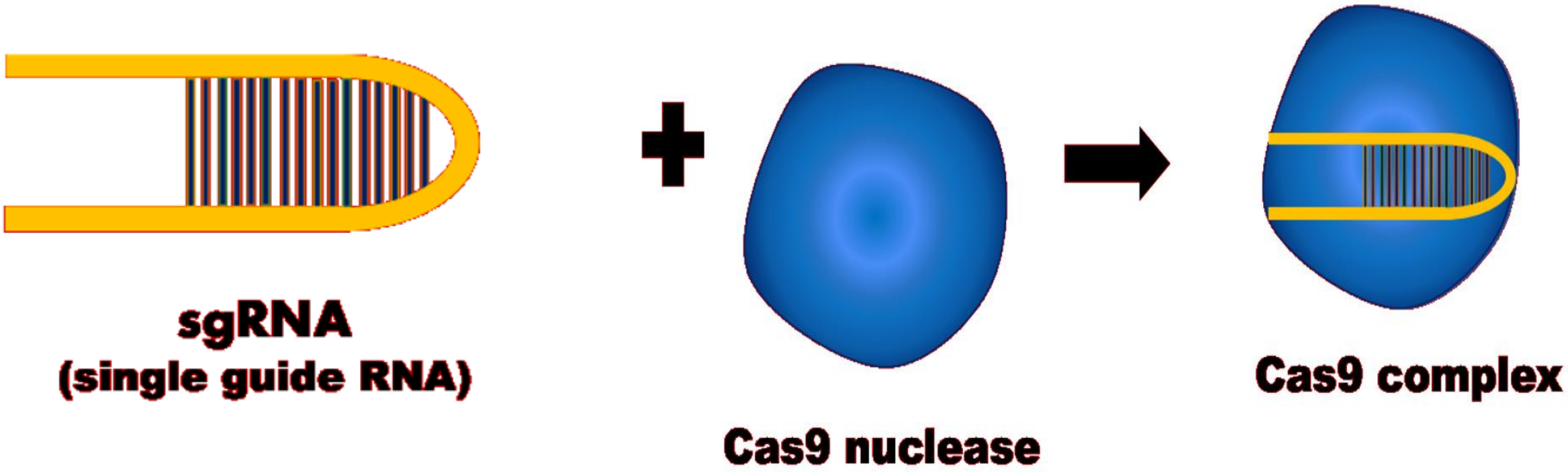
Broad, Caribou LIC

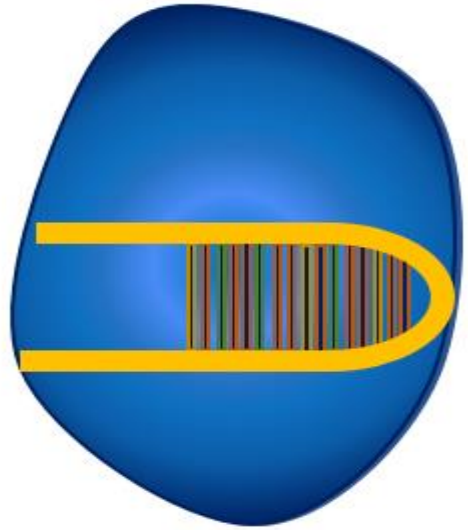
TACONIC

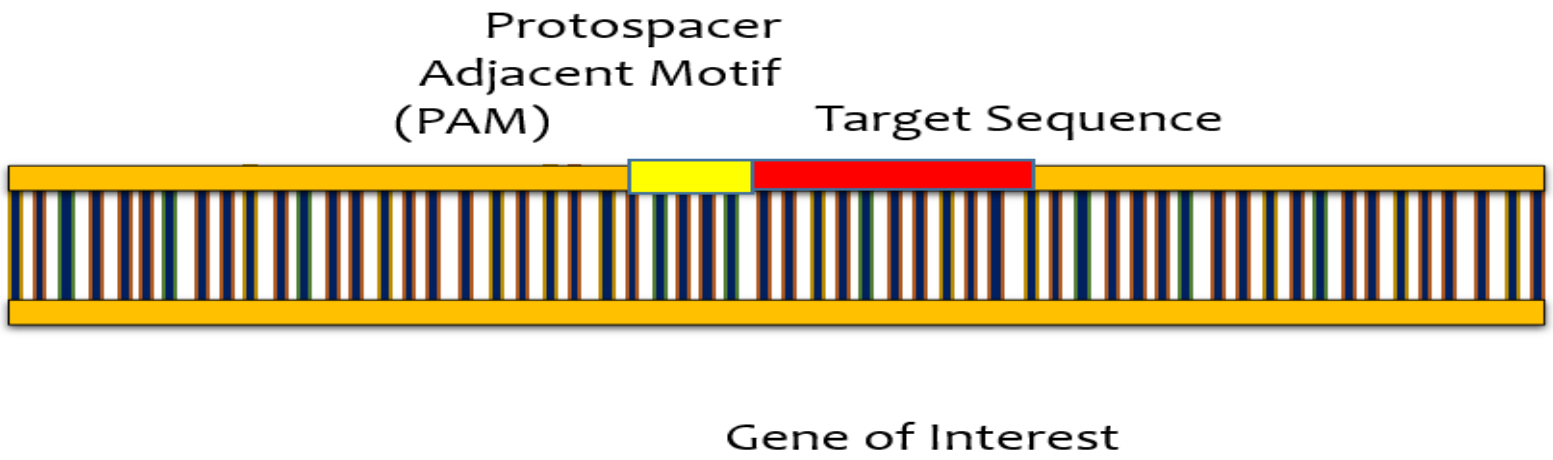
Broad LIC

Second: Cas9

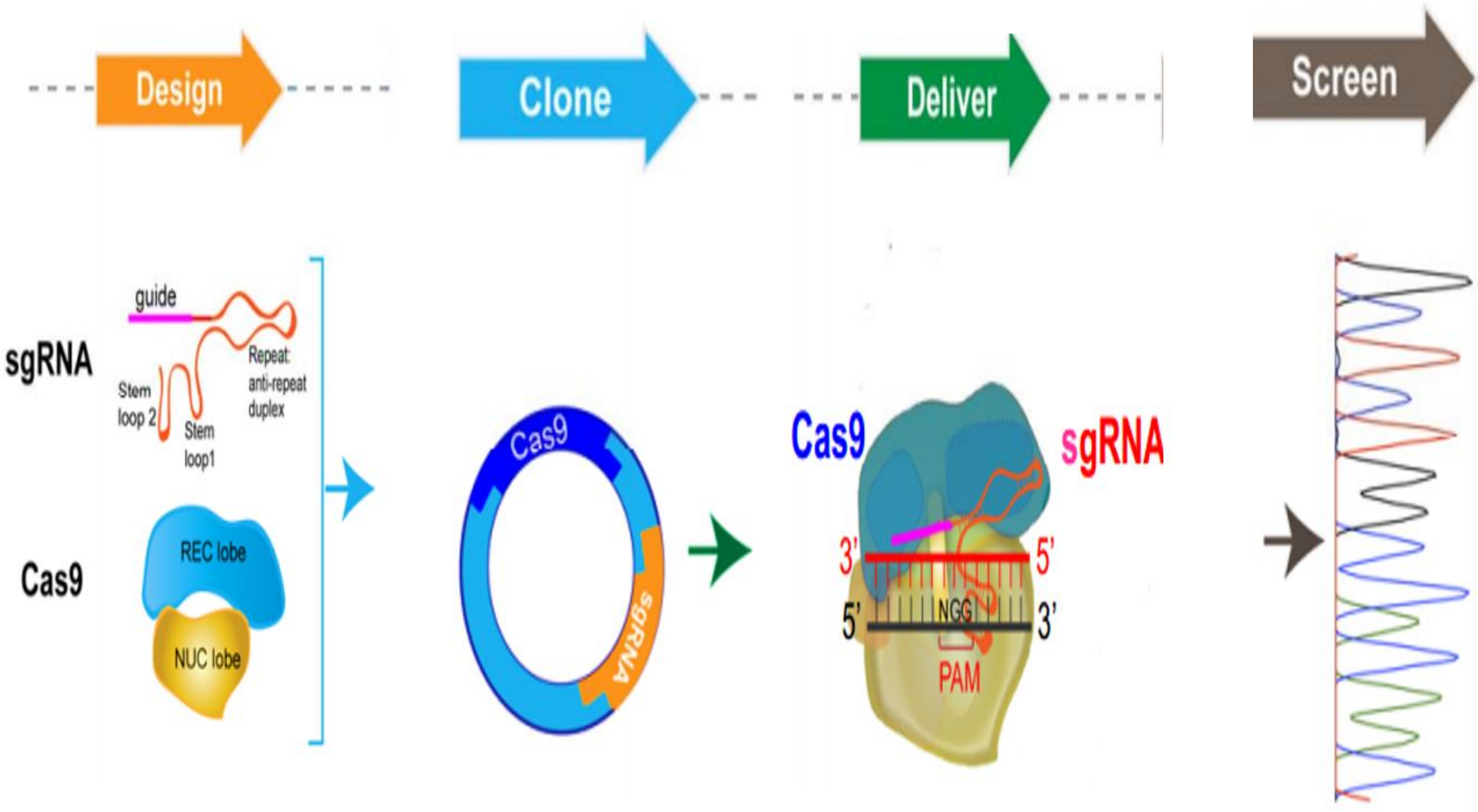


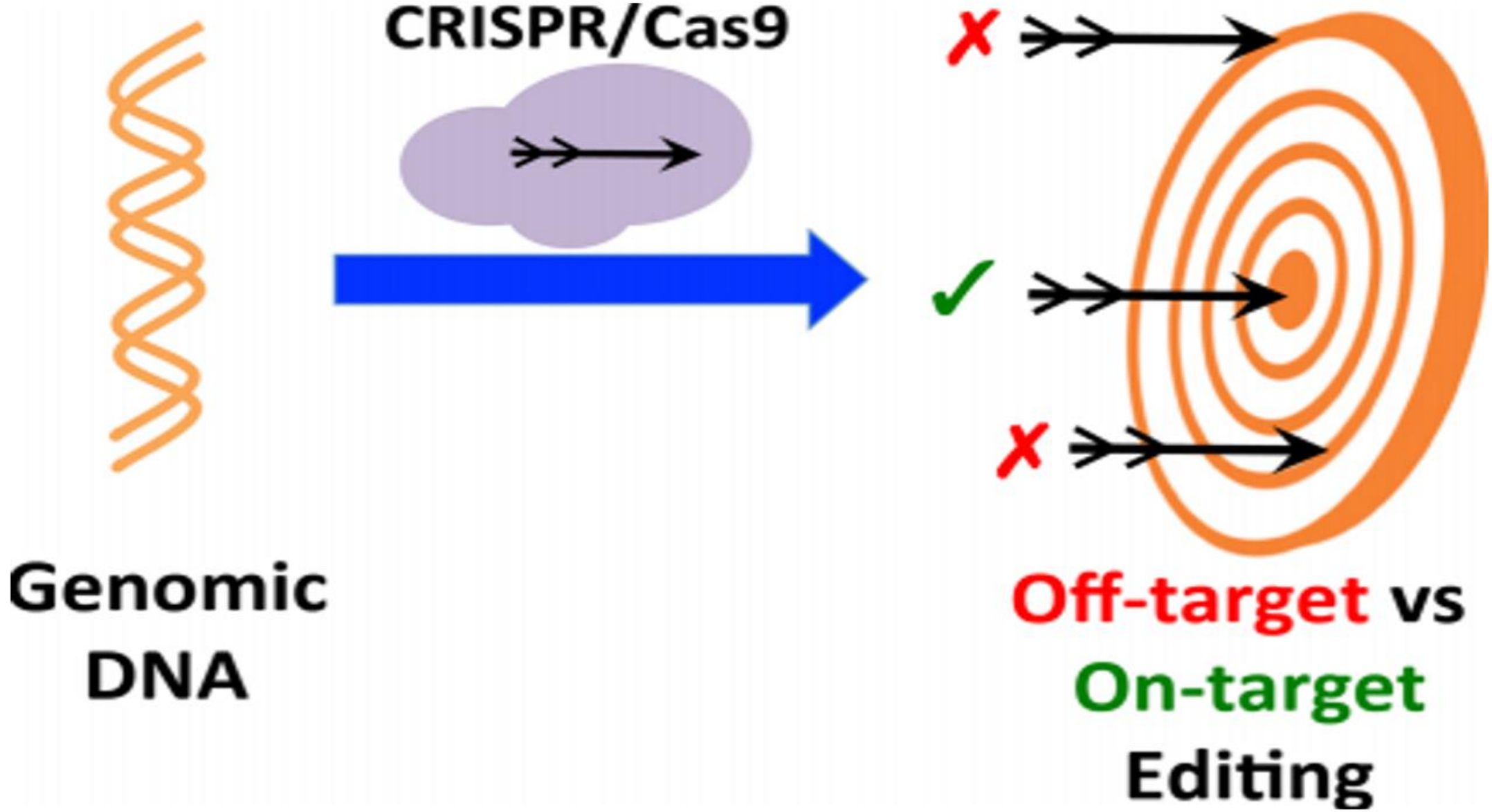

sgRNA
(single guide RNA)
Cas9 nuclease
Cas9 complex



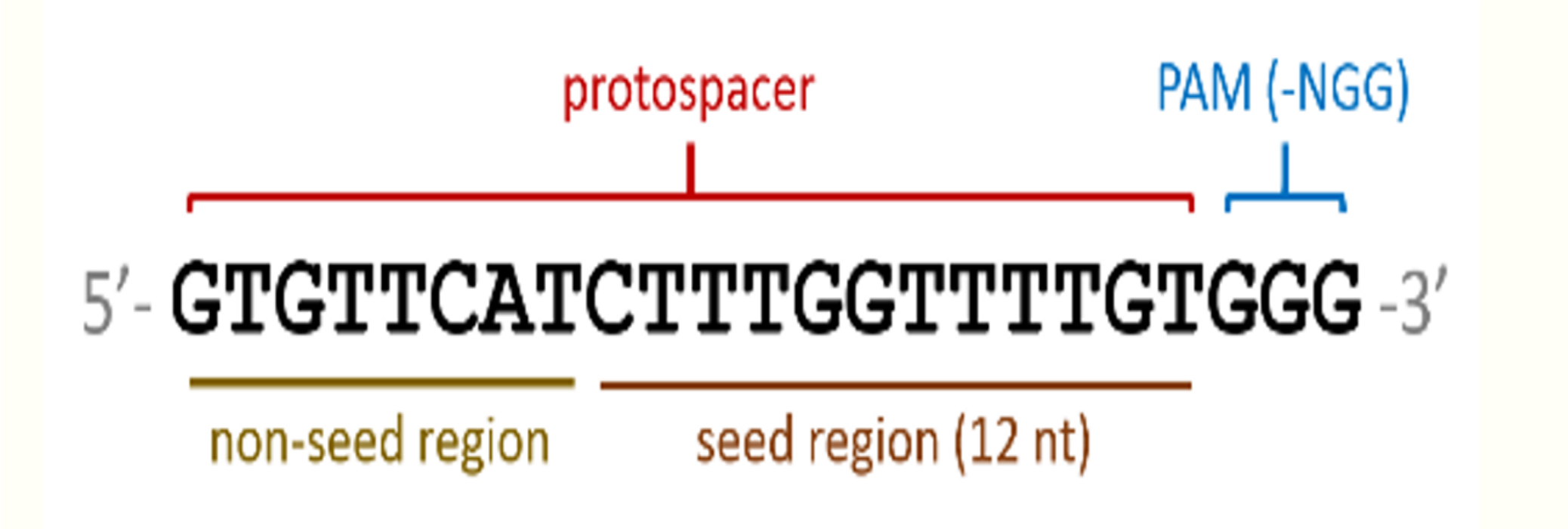

Protospacer
Adjacent Motif
(PAM)
Target Sequence
Gene of Interest

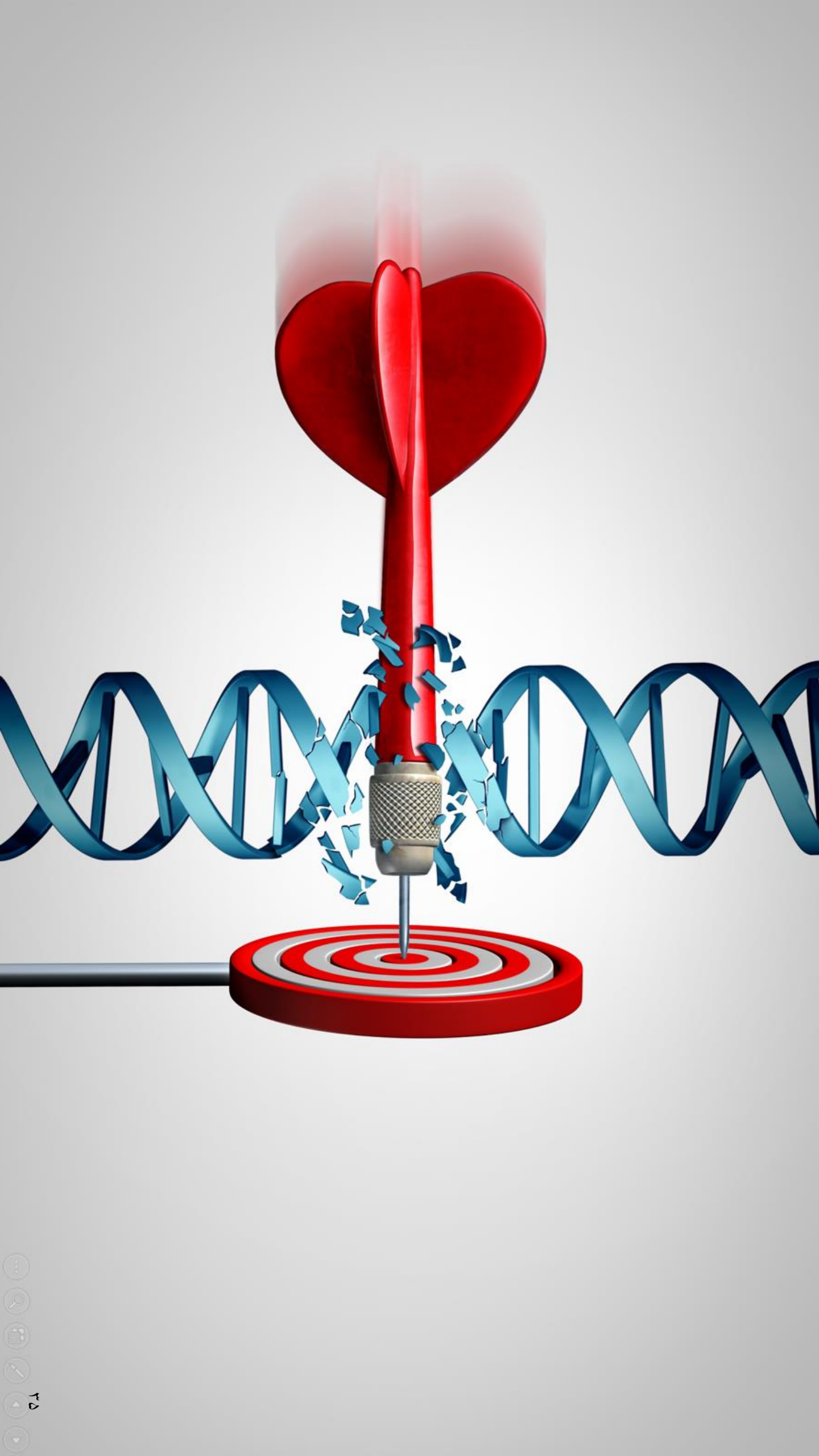
Cas Variant	PAM Sequence
SpCas9	NGG
SpCas9 VRER Variant	NGCG
SpCas9 EQR Variant	NGAG
SpCas9 VQR Variant	NGAN or NGNG
SaCas9	NNGRRT
Cpf1	TTN


Design
Clone
Deliver
Screen
sgRNA
guide
Repeat:
anti-repeat
duplex
Stem
loop 2
Stem
loop1
Cas9
REC lobe
NUC lobe
Cas9
sgRNA
Cas9
sgRNA
3'
5'
5'
3'
NGG
PAM


CRISPR/Cas9
Genomic DNA
Off-target vs
On-target
Editing

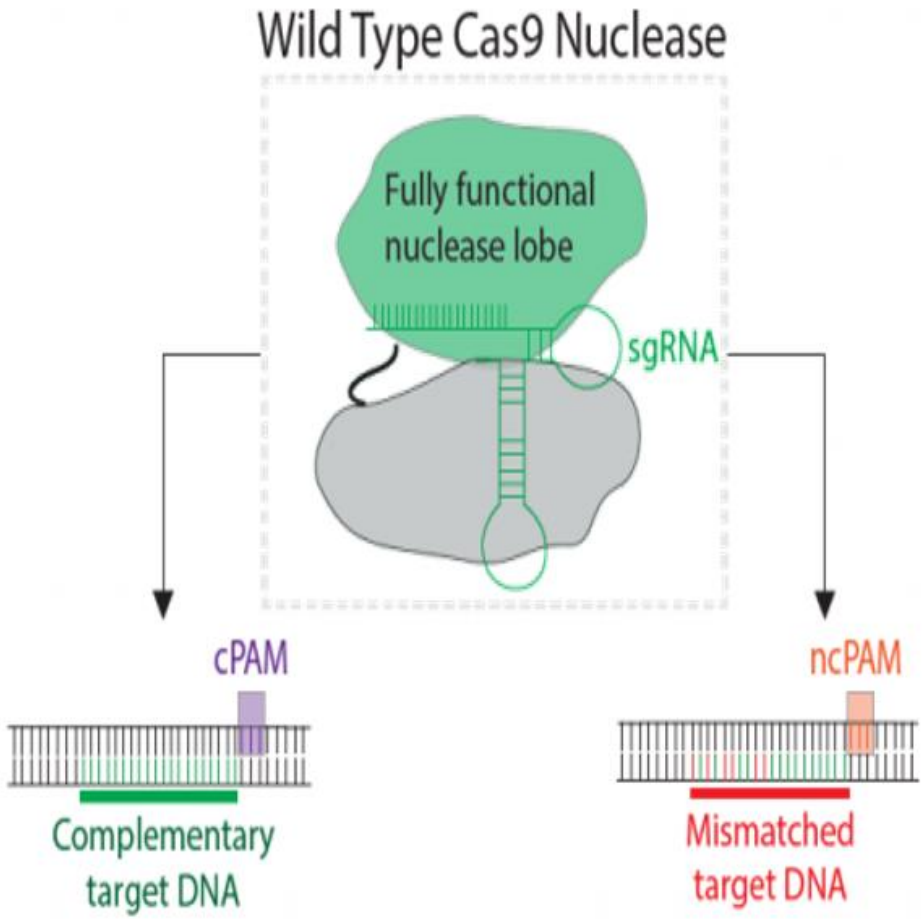

?
Solution

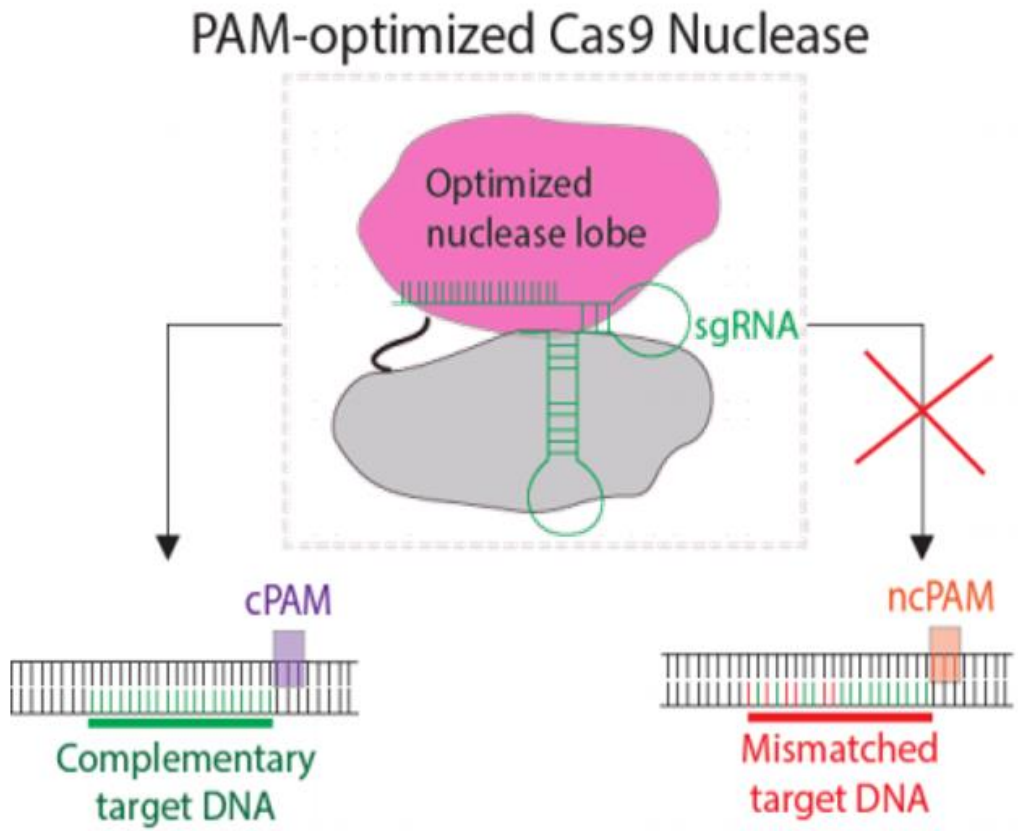

protospacer
PAM (-NGG)
5′- GTGTTCATCTTTGGTTTTTGTGGG -3′
non-seed region
seed region (12 nt)

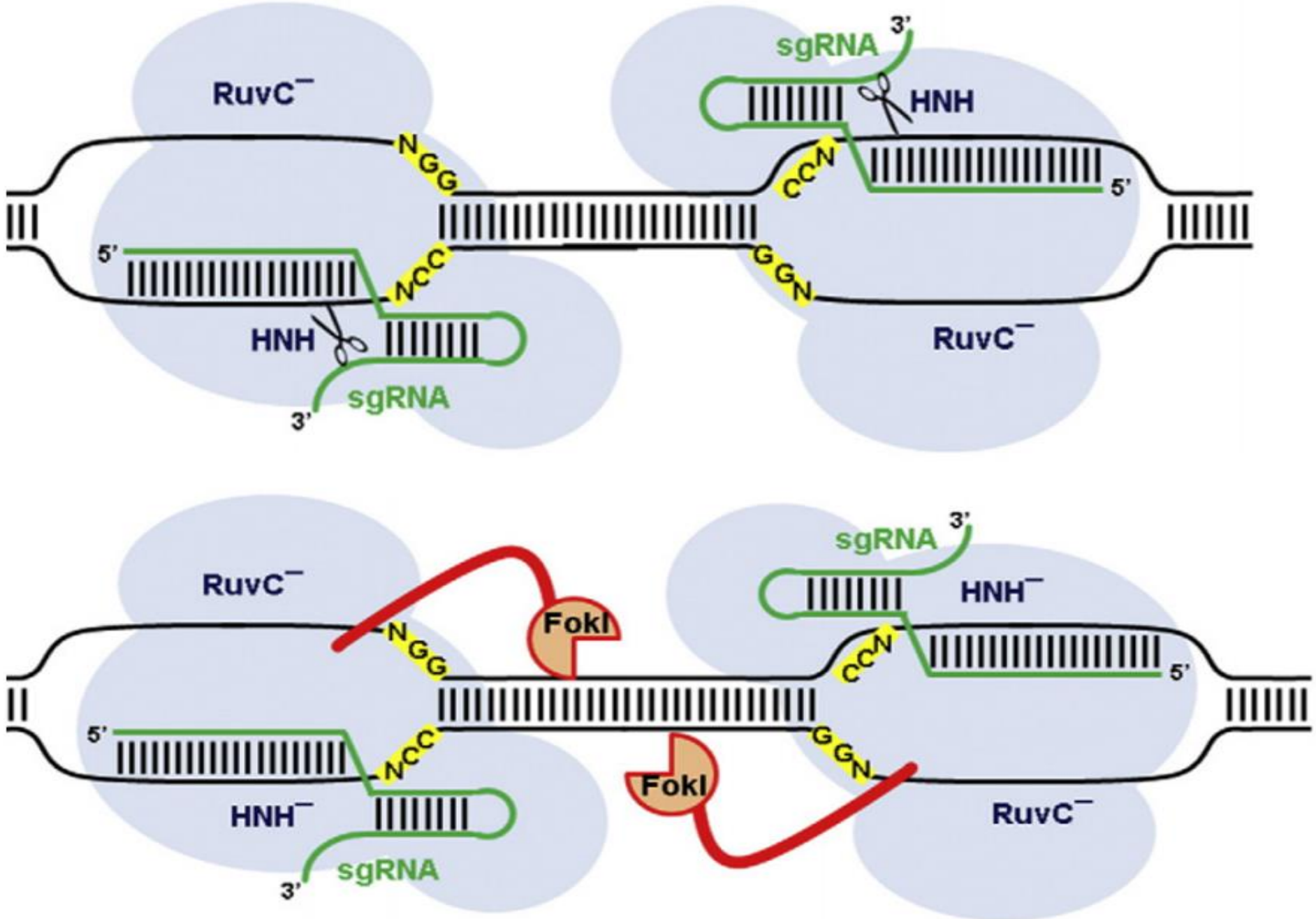


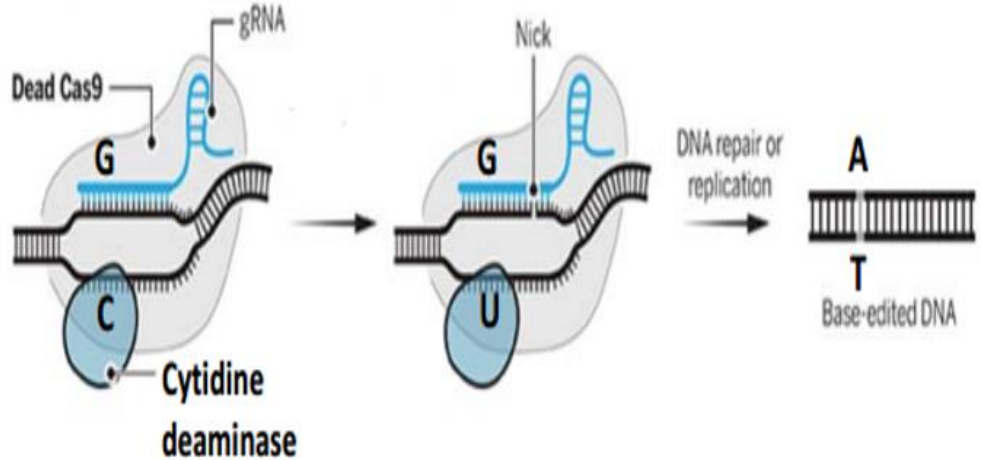

ON-TARGET ACTIVITY


SPECIFICITY


Wild Type Cas9 Nuclease
Fully functional nuclease lobe
sgRNA
cPAM
ncPAM
Complementary target DNA
Mismatched target DNA

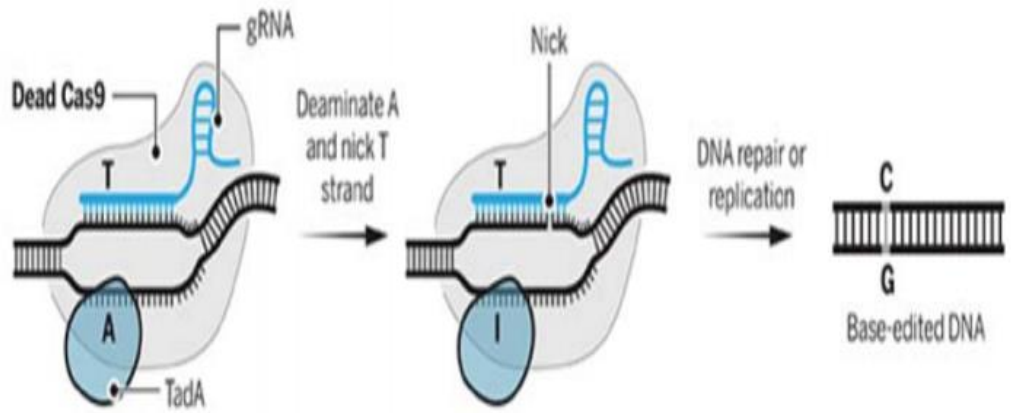

PAM-optimized Cas9 Nuclease
Optimized nuclease lobe
sgRNA
cPAM
ncPAM
Complementary target DNA
Mismatched target DNA


RuvC⁻
NGG
5'
NCC
HNH
sgRNA
3'
sgRNA
3'
HNH
CCN
5'
GGN
RuvC⁻
RuvC⁻
FokI
NGG
5'
NCC
HNH⁻
sgRNA
3'
sgRNA
3'
HNH⁻
CCN
5'
GGN
FokI
RuvC⁻

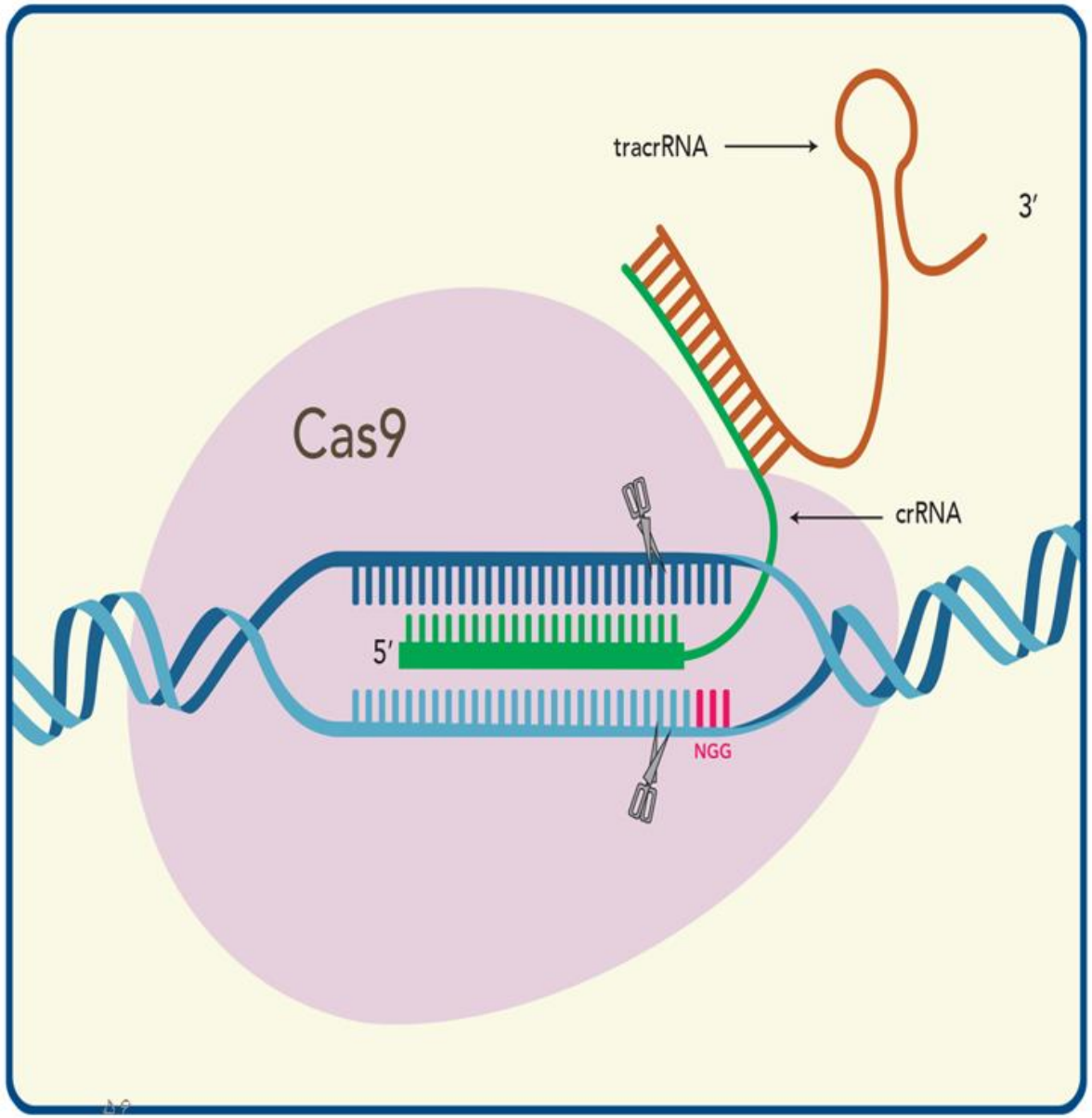


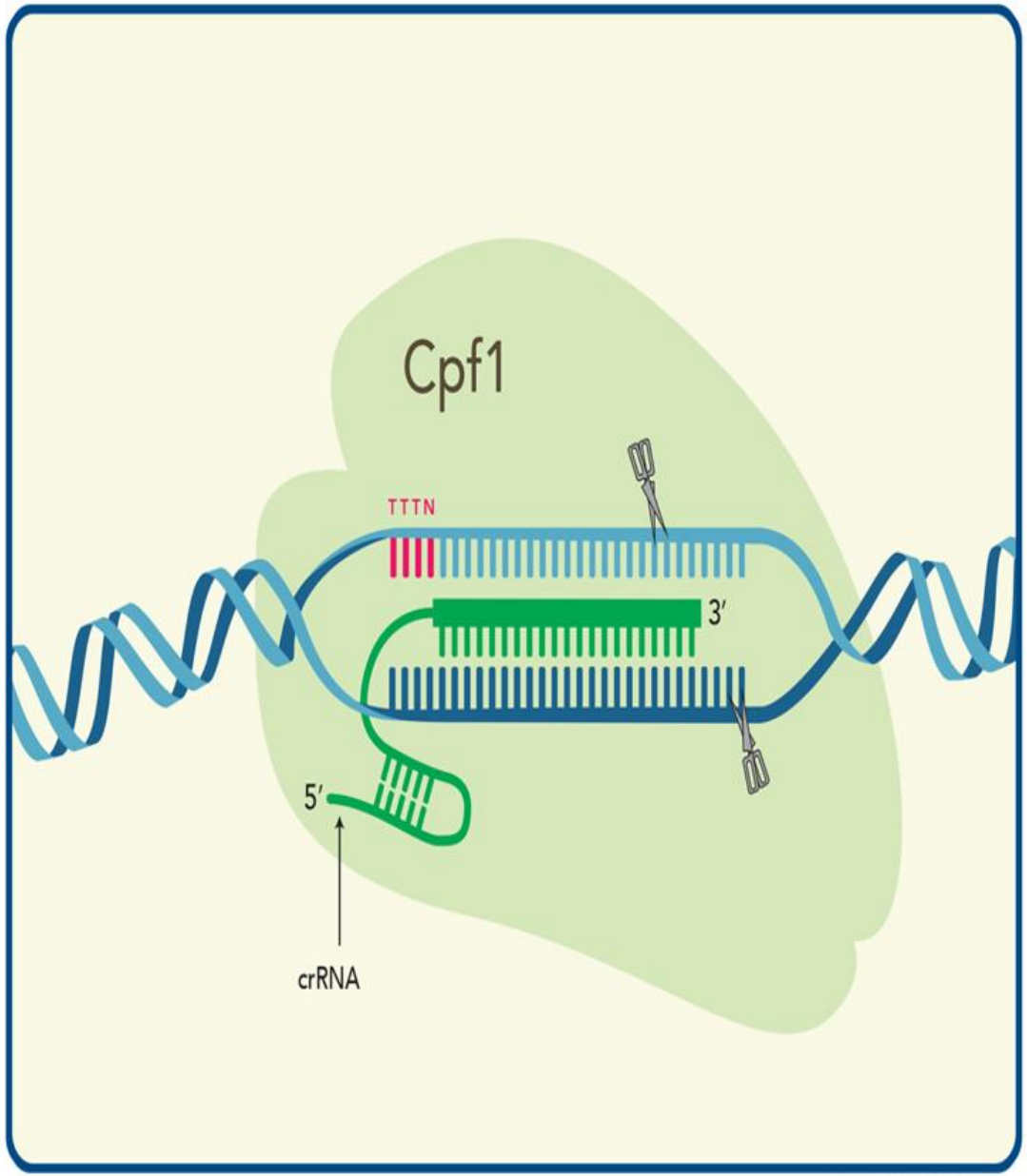
Base editors:

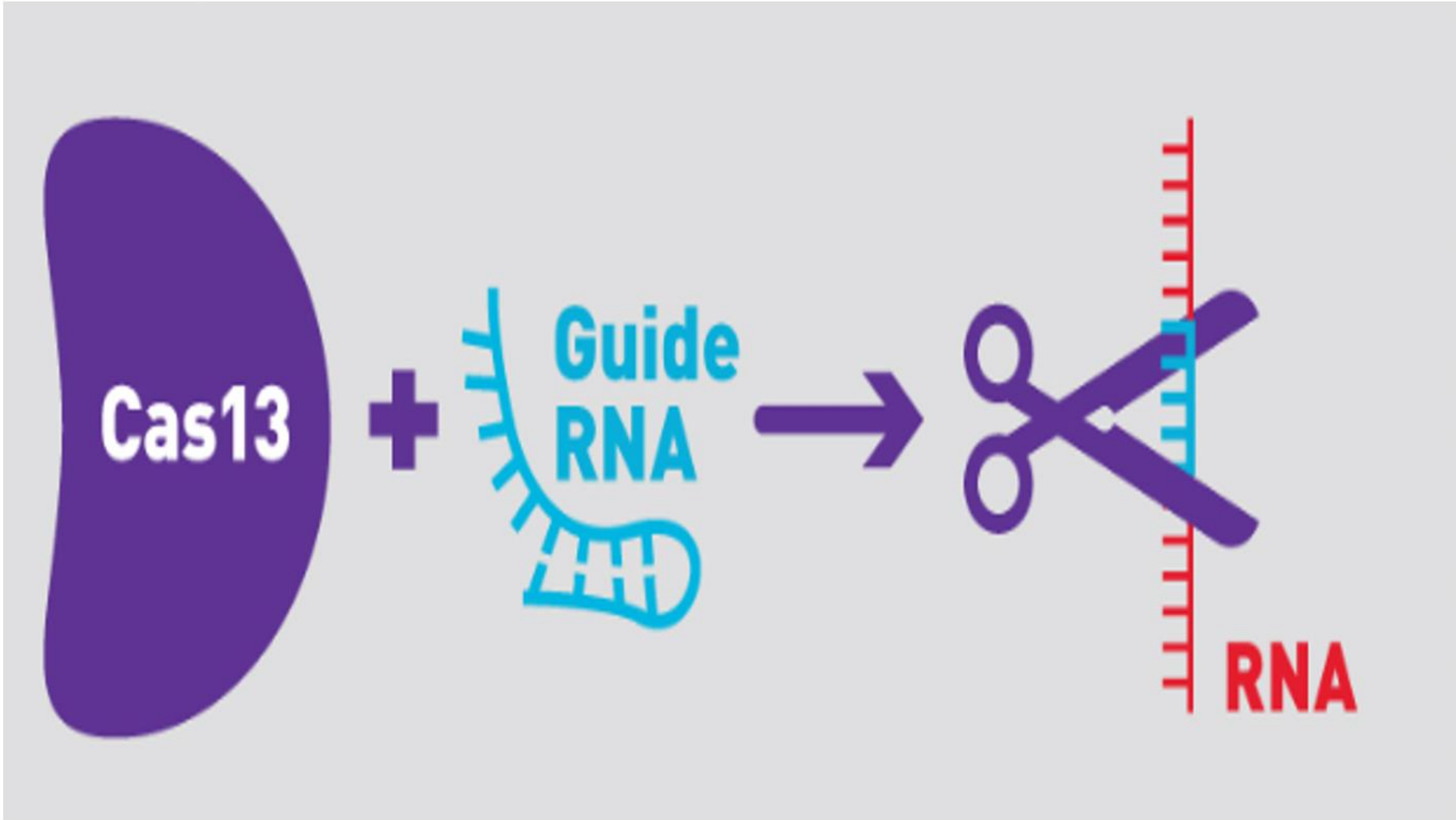
dCas9-cytidine deaminase (C-> T; G-> A)



dCas9-TadA (A-> G; T-> C)

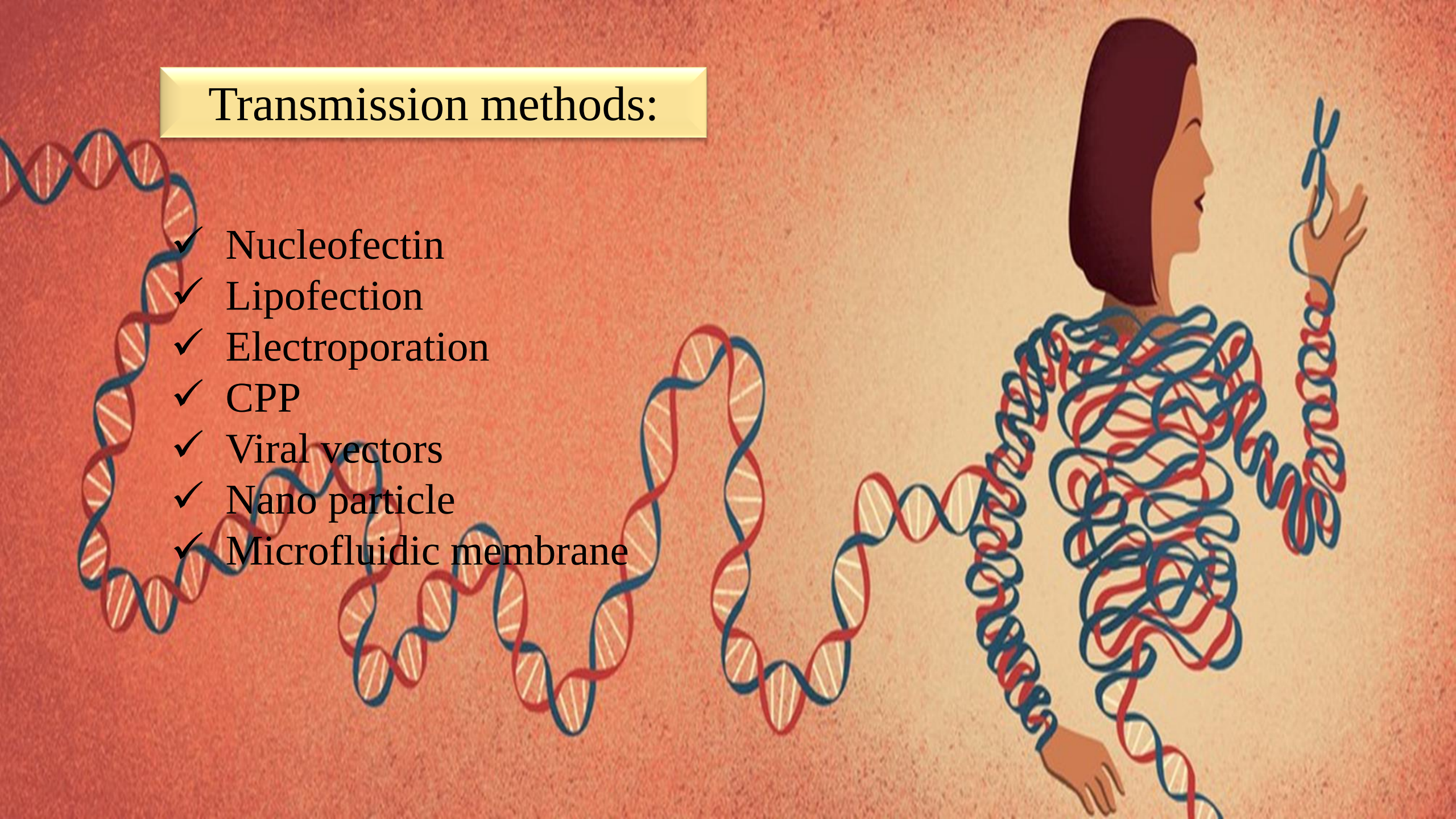

tracrRNA
3′
Cas9
crRNA
5′
NGG


Cpf1
TTTN
3′
5′
crRNA


Cas13
Guide
RNA
RNA

Transmission methods:

- ✓ Nucleofectin
- ✓ Lipofection
- ✓ Electroporation
- ✓ CPP
- ✓ Viral vectors
- ✓ Nano particle
- ✓ Microfluidic membrane



Off-target detection approaches

- In silico prediction
- In vitro assessment
- In situ (cell) assessment

Selected in silico prediction assays

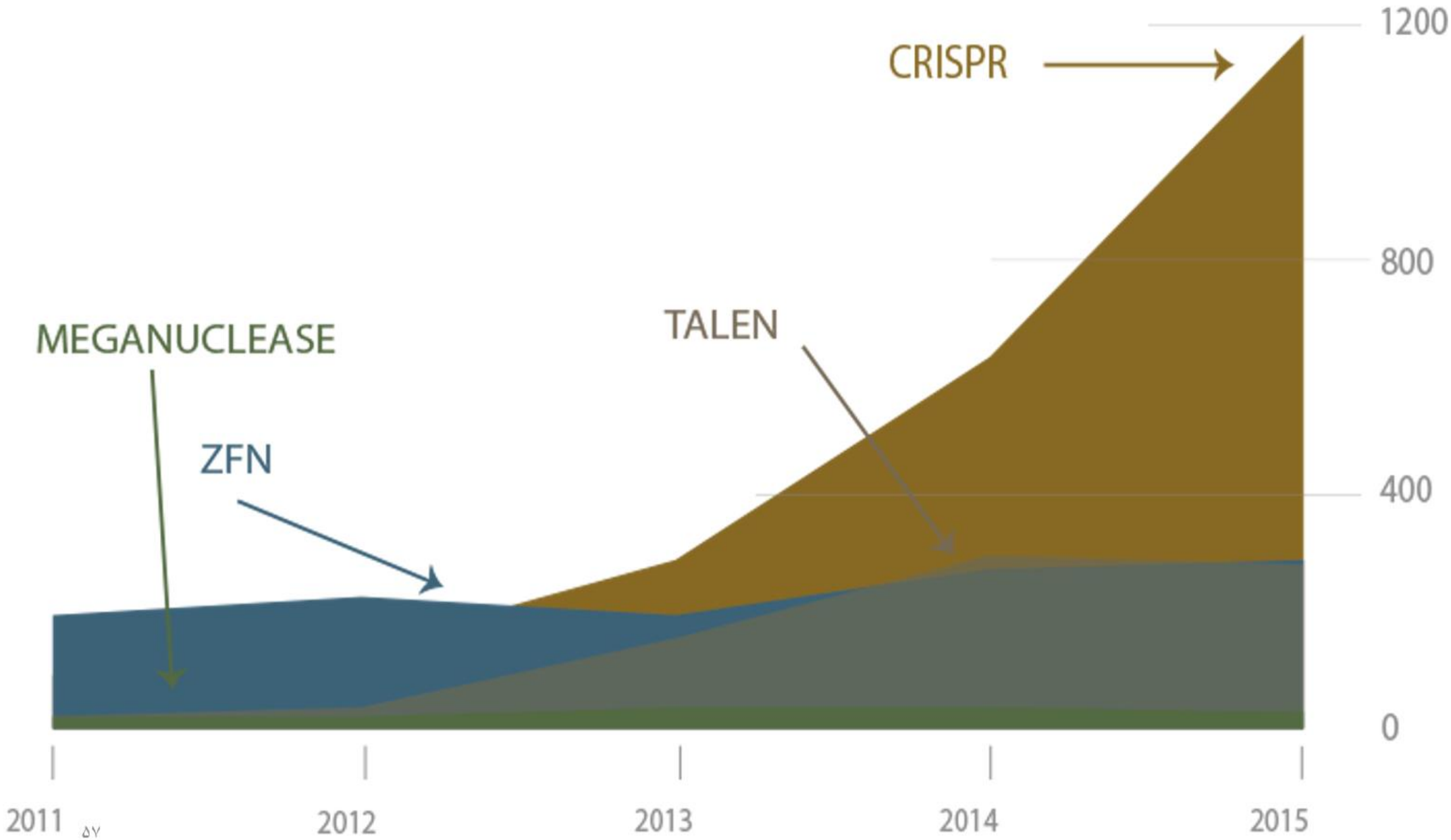
Selected in silico predidiction assays	Resources
Cas-OFFinder	http://WWW.rgenome .net/cas-offinder
CRISPR Design Tool	http://crisp.mit.edu/
CasFinder	http://arep.med.harvard.edu/CasFinder
E-CRISP	http://WWW.e-crisp.org/E-CRISP/
Breaking-cas	http://bioinfogp.cnb.csic.ec/tools/breakingcas/
CRISPOR	http://crispor.tefor.net
CHOPCHOP	http://chopchop.cbu.uib.no

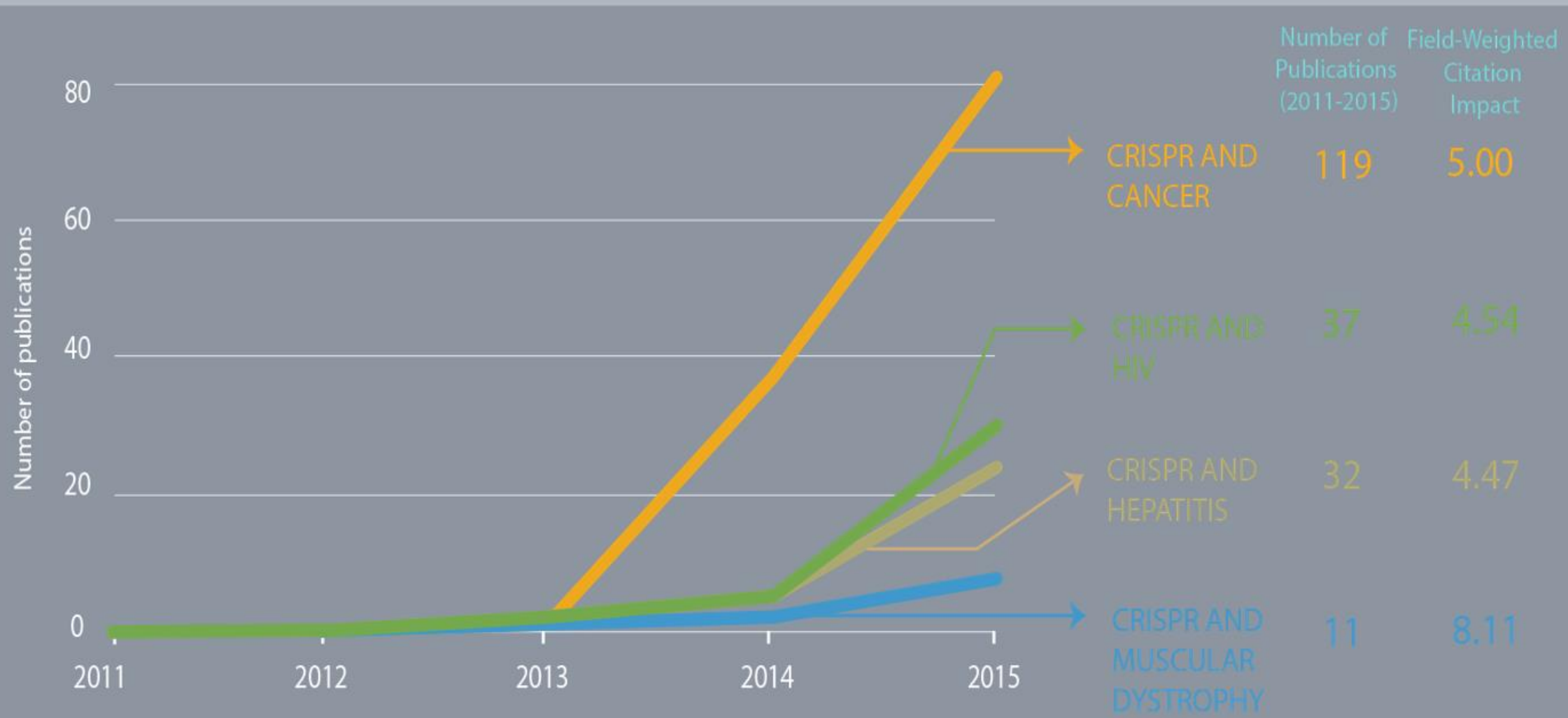
In vitro genome-wide assays

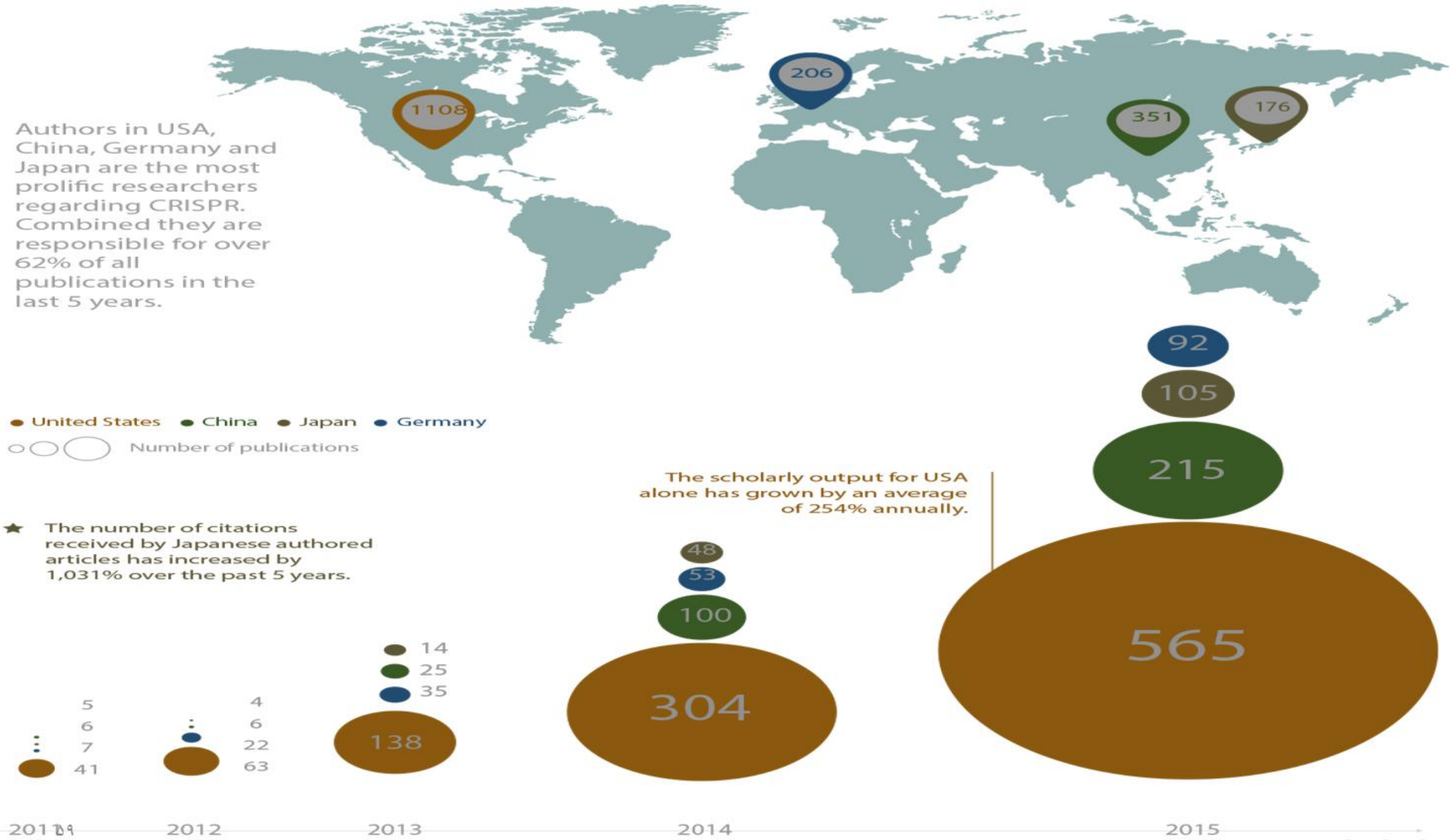
Selected in vitro genome-wide assays	Description
Digenome-Seq	Purified genomic DNA is digested with a nuclease and subjected to whole genome sequencing .Off targets are computationally identified
CIRCLE-Seq	Purified genomic DNA is sheared and circularized, and residual linear DNA us degraded. The Cas9 nuclease is used to linearize circular DNA containing a Cas9 cleavage site, and the cleaved ends are PCR-amplified and sequenced to identify off-targets.
SITE-Seq	Purified genomic DNA is cleaved using Cas9, and Cas9 cleavage sites are biochemically tagged and enriched . Next-gene sequencing and bioinformatics analysis is then used to identify off-targets cleavage sites.

Cell-based genome-wide assays

Selected cell-based genome-wide assays	Description
GUIDE-Seq	Double-stranded breaks created by the Cas9 nuclease are tagged using small double-stranded oligonucleotides, PCR amplified, and sequenced to map the double-stranded breaks
LAM-HTGTS	Chromosomal translocations of off-target and on-target breaks are PCR amplified and analyzed by next-gene sequencing
BLISS	Double-stranded breaks are biochemically labeled, and their downstream sequences are amplified using in vitro transcription and analyzed using next-gene sequencing.

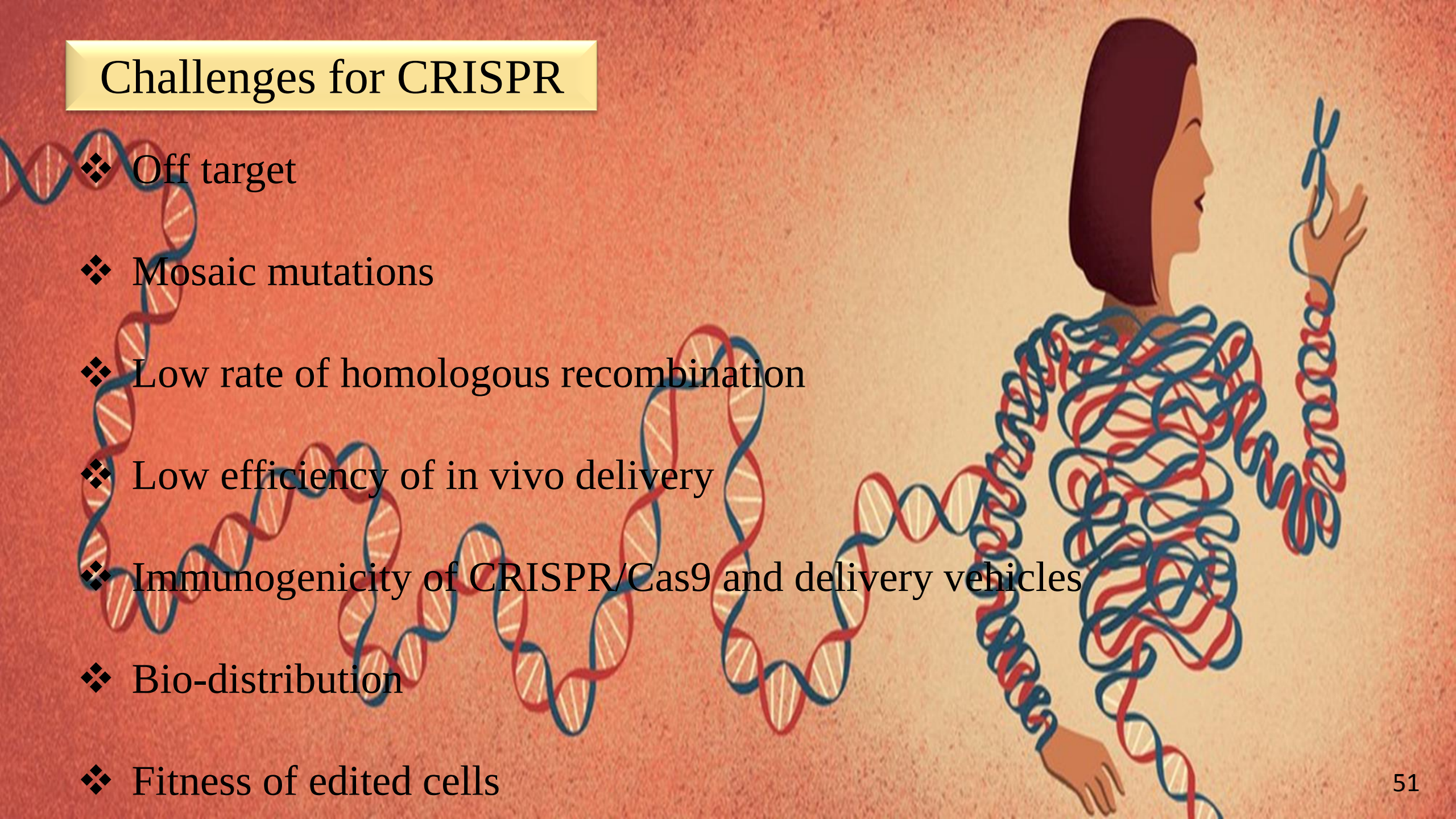

1200
800
400
0
CRISPR
TALEN
ZFN
MEGANUCLEASE
2011
2012
2013
2014
2015


Number of publications
80
60
40
20
0
2011
2012
2013
2014
2015
Number of Publications (2011-2015)
Field-Weighted Citation Impact
CRISPR AND CANCER
119
5.00
CRISPR AND HIV
37
4.54
CRISPR AND HEPATITIS
32
4.47
CRISPR AND MUSCULAR DYSTROPHY
11
8.11

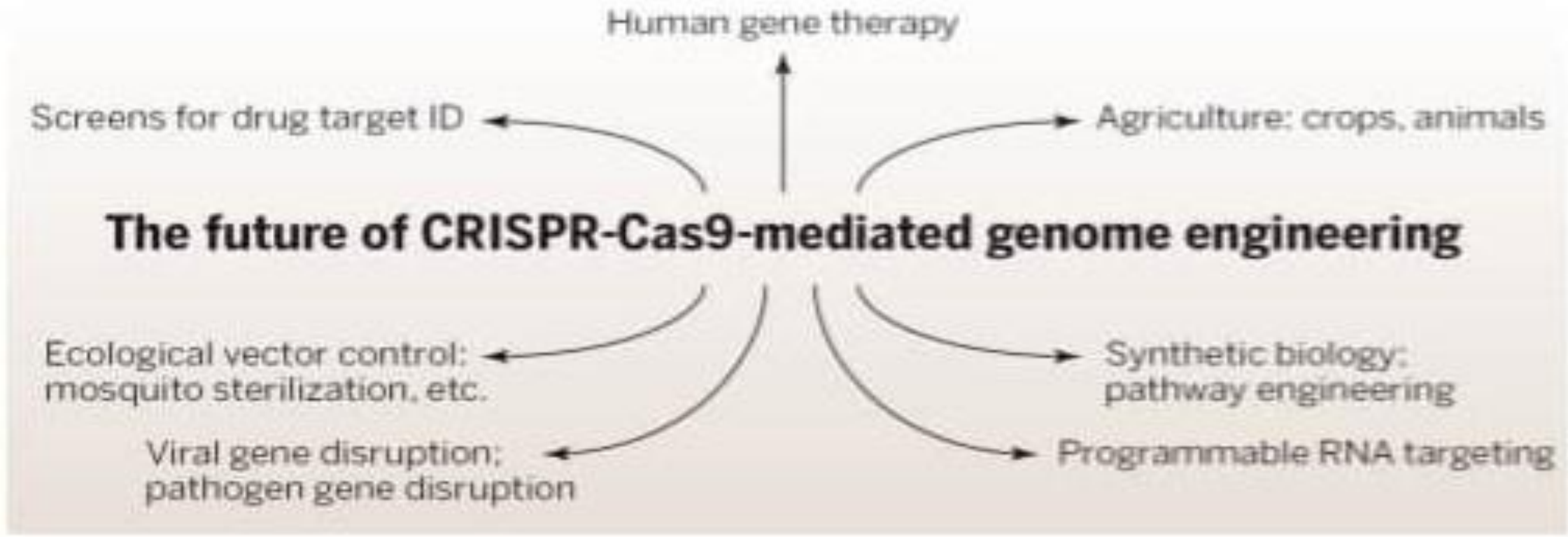

Authors in USA, China, Germany and Japan are the most prolific researchers regarding CRISPR. Combined they are responsible for over 62% of all publications in the last 5 years.
1108
206
351
176
United States
China
Japan
Germany
Number of publications
The number of citations received by Japanese authored articles has increased by 1,031% over the past 5 years.
The scholarly output for USA alone has grown by an average of 254% annually.
5
6
7
41
4
6
22
63
14
25
35
138
48
53
100
304
92
105
215
565
2011
2012
2013
2014
2015

Challenges for CRISPR

- Off target
- Mosaic mutations
- Low rate of homologous recombination
- Low efficiency of in vivo delivery
- Immunogenicity of CRISPR/Cas9 and delivery vehicles
- Bio-distribution
- Fitness of edited cells



Future applications in biomedicine and biotechnology



Source: Jennifer A. Doudna and Emmanuele Charpentier, The new frontier of genome engineering with CRISPR-Cas9 , ScienceMag2014, VOL 346 ISSUE 6213

Software and Databases

CRISPR direct

Rational design of CRISPR/Cas target.

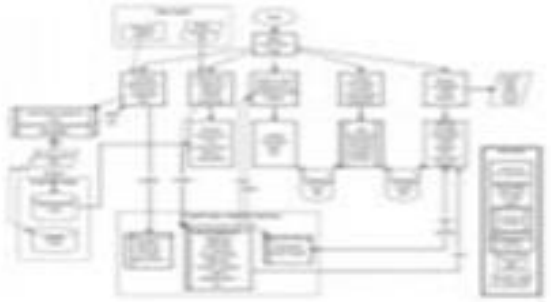
addgene

The nonprofit plasmid repository

E-CRISP

Design of CRISPR constructs

CasFinder



GeCKO

Genome-scale CRISPR Knock-Out

TOP

ZiFiT Targeter

Figures: Software data bases and Publications

Therapeutic CRISPR applications

- Cell (line) engineering
- Genetically animal models

Preclinic

- Diseases gene therapy (editing)
  - Somatic cells (ex vivo/in vivo)
  - Germ cells (ethics)
  - Embryo (ethics)

Clinic

The potential of CRISPR-Cas9 for treating genetic disorders

Disease	Target gene	Transmission method	Target cell	Efficiency level(%)
Beta-thalasemia	Gene deletion HHB	Electroporation	Human ipsc cells,human zygote	17.6 14.3
Cystic fibrosis	Gene deletion CFTR	Lipofection	Human organoid intestine cells	…..
HIV-1	Inactivation DNA of provirus	Electroporation	Human ipsc cells, JL.at10.6 cells	100 30
DMD	Exon deletion in distrophin gene	Electroporation	Human ipsc cells	50

Disease	Target gene	Transmission method	Target cell	Efficiency level(%)
Tyrosinemia(type 1)	Point mutation in FAH gene	Hydrodynamic injection	Human ipsc cells	0.4
AAT Defficiency	Point mutation in SERPINA1	Electroporation	Human ipsc cells	18.8
Polycythemia-vera	Point mutation in JAK2	Electroporation	Human ipsc cells	9.15
Cataract	Deletion of crygc	Electroporation	Spermatogonic & stemcells of the mouse	29.7
LDL-C	Deletion of pcsk9	AAV	Mouse liver in vitro cells	50 40

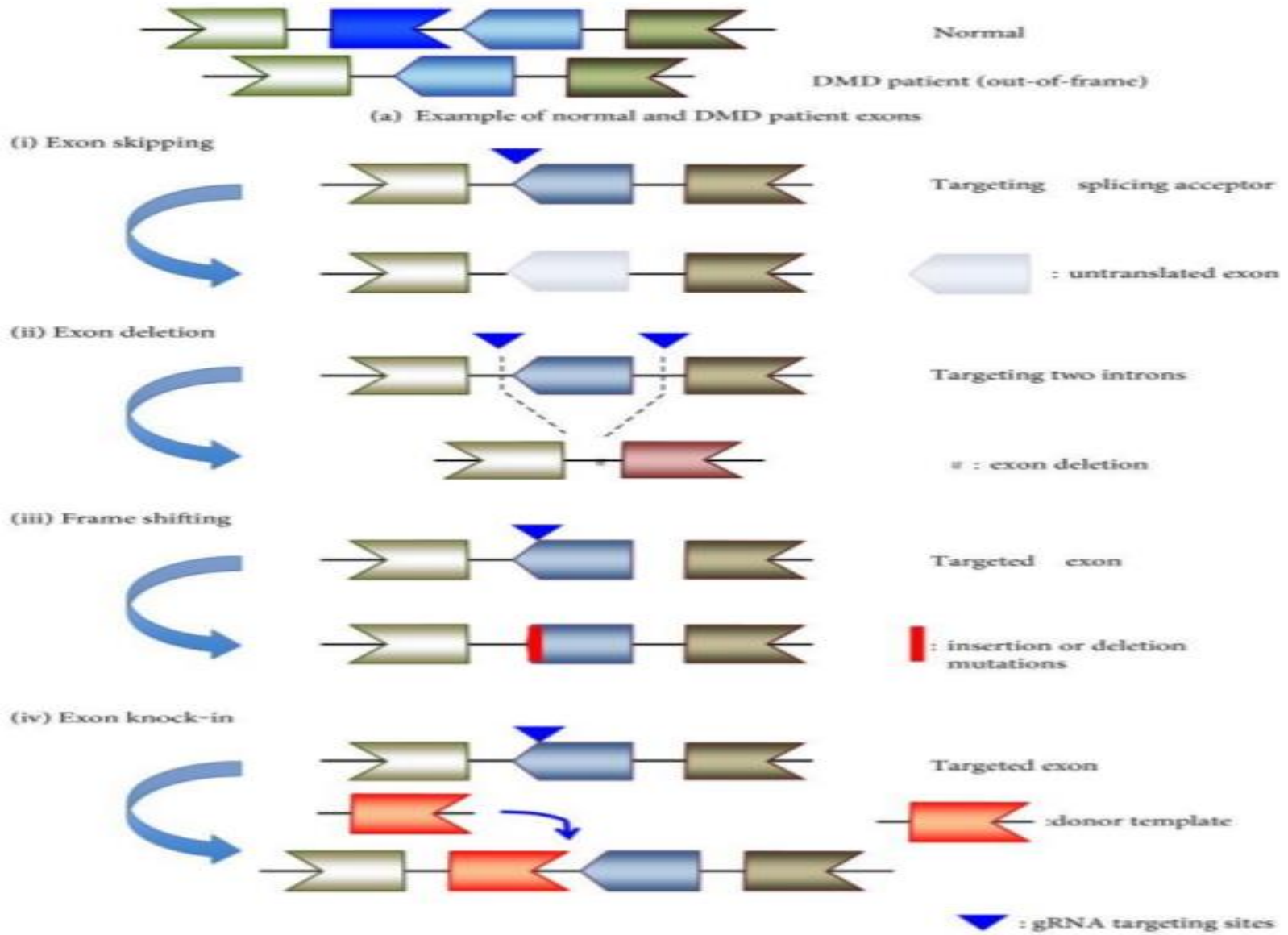
What is Duchenne muscular dystrophy ?

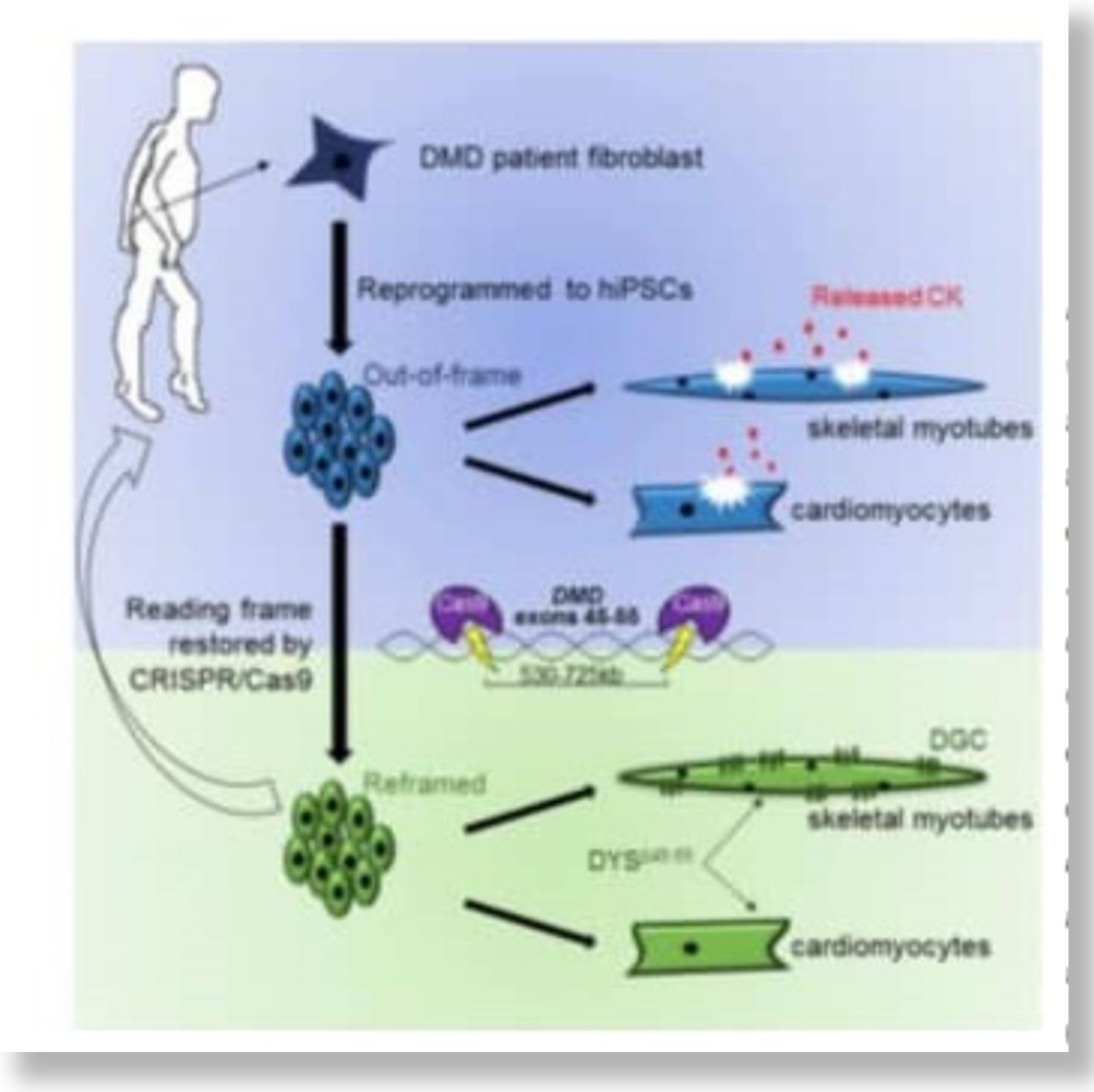
- Is a fatal X-linked recessive neuromuscular disease prevalent in 1 in 3500 to 5000 males world wide
- Progressive muscle weakness
- Defects in muscle proteins
- Death of muscle tissue

More symptoms of DMD

- Muscle weakness
- Delayed developmental milestones
- Difficulty rising (Gower’s sign)
- Difficulty walking/running

DMD-mutation targeting strategies by CRISPR-Cas9





Ex-vivo gene therapy with CRISPR-Cas9 in autologous stem cells.

Fibroblasts are derived from a DMD patient affected by an out-of-frame mutation. Subsequently , fibroblasts are reprogrammed into ipscs. CRISPR-Cas9 corrects the ORF of ipscs by using an multi-exon deletion approach. Corrected ipscs are differentiated into muscle stem cells and transplanted back into the patient.

Journal of *Personalized Medicine*

MDPI

Review

Applications of CRISPR/Cas9 for the Treatment of Duchenne Muscular Dystrophy

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Abstract: Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive neuromuscular disease prevalent in 1 in 3500 to 5000 males worldwide. As a result of mutations that interrupt the reading frame of the *dystrophin* gene (*DMD*), DMD is characterized by a loss of dystrophin protein that leads to decreased muscle membrane integrity, which increases susceptibility to degeneration. CRISPR/Cas9 technology has garnered interest as an avenue for DMD therapy due to its potential for permanent exon skipping, which can restore the disrupted *DMD* reading frame in DMD and lead to dystrophin restoration. An RNA-guided DNA endonuclease system, CRISPR/Cas9 allows for the targeted editing of specific sequences in the genome. The efficacy and safety of CRISPR/Cas9 as a therapy for DMD has been evaluated by numerous studies in vitro and in vivo, with varying rates of success. Despite the potential of CRISPR/Cas9-mediated gene editing for the long-term treatment of DMD, its translation into the clinic is currently challenged by issues such as off-targeting, immune response activation, and sub-optimal in vivo delivery. Its nature as being mostly a personalized form of therapy also limits applicability to DMD patients, who exhibit a wide spectrum of mutations. This review summarizes the various CRISPR/Cas9 strategies that have been tested in vitro and in vivo for the treatment of DMD. Perspectives on the approach will be provided, and the challenges faced by CRISPR/Cas9 in its road to the clinic will be briefly discussed.

THE KAVLI PRIZE

He Jiankui says he is proud of the work that has led to the birth of genetically changed twins Nana and Lulu.

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1.Bayat H , Naderi F, Mohammadian O, Rahimpour A. The CRISPR-Cas system and Recent Advances in Its Precision Performance. Journal of Jiroft University of Medical Sciences 2017;3(1):23-28.

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Academic year : 2016-2018

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5.Kenji Rowel Q.Lim, Chantal yoon, and Toshifumi Yokta. Applications of CRISPR/Cas9 for the treatment of Ducchenne Muscular Dystrophy. Published:24 November 2018 .

6.Xiao-Hui Zhang, Louis Y Tee, Xia-Gang Wang, Qun-shan Haung and Shi-Hua Yang . Off-target Effects in CRISPR-Cas9-mediated genome Engineering.Mulecular Therapy –Nucleic acids (2015),DOI:10.1038/mtna.2015.37


Thank
you